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MASTER OF SCIENCE

Exploring allosteric pockets in protein kinases a multidisciplinary approach

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Exploring allosteric pockets in protein kinases:
a multidisciplinary approach

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MRes Biological Chemistry and Drug Discovery

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Declaration

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1. Introduction

The human kinome is a vast domain of the human genome that plays a key role in the correct functioning of the organism's cellular signalling processes. The aberrant signalling of protein kinases is found in many cancers, and genetic aberrations are a common cause for metabolic and autoimmune disorders. The fact that a number of drugs targeting these proteins are approved is not a coincidence. However protein kinase drug discovery has not been particularly efficient relative to the resources expended on the challenge. Kinases are particularly challenging for traditional inhibitors due to the high conservation among families and subtypes in their active sites, making selectivity an important issue. Hence, there is an increasing opportunity of therapeutics whose mechanism of action is independent of the active site. In this multidisciplinary project, the possibility of rationally designing allosteric compounds was tackled three-dimensionally: via computational, biophysical and biochemical methods.

1.1. Background on protein kinase research and aims of the project

Phosphorylation is one of the main post-transcriptional modifications that take place in the cell. It is a process in which phosphate groups are added to proteins via the usage of Adenosine triphosphate (ATP) on serines, threonines or tyrosines, substrates to this reaction (*Cohen, 2002*). Protein kinases are the enzymes that catalyse this reaction and can be broadly classified into Ser/Thr and Tyrosine kinases, with some instances of dual-specificity kinases (*Hanks and Hunter, 1995*). In the human genome, approximately 2% percentage of the genes encoding kinases expressing around 500 genes.

Kinases are attractive therapeutic targets due to their essential and ubiquitous role in cell signalling and cellular processes, and they are mostly involved in cancer and autoimmune diseases. For this reason, the search for adequate kinase inhibitors has garnered much attention in the past years.

There are a large number of kinases that are currently targets for the pharmaceutical industry (*Fedorov et al. 2010*). Although approved therapeutics that target these proteins successfully exist, there are several issues with the currently available drugs. The first and most troublesome one is that of selectivity. Due to the evolutionarily essential and thus highly conserved active site, drug molecules that target one protein

kinase are likely to also affect those of the same family and sometimes exhibit cross-family activity. As a consequence, this can produce an array of side effects that can be detrimental, and sometimes exacerbate the disease being treated (*Kang et al. 2013*). A possible solution for this problem would be the use of drugs with allosteric mechanism of action, activity of which is given by the binding to an alternative, less conserved site in order to either enhance, inhibit, or modulate the activity of the kinase. Such drugs already exist in the market, and some are in clinical trials. An example of this is the widely used Gleevec, an allosteric drug which inhibits the activity of the aberrant tyrosine kinase Bcr-Abl, expressed in patients with chronic myeloid leukaemia (*Hantschel, 2012*).

There are three types of kinase inhibitors: Type I, which recognise the active conformation of a kinase and bind to ATP-binding site commonly by several hydrogen bonds, resulting in competitive binding with ATP (e.g. Sunitinib); type II, where the drug recognises the inactive conformation of kinase and indirectly competes with ATP by occupying the hydrophobic pocket adjacent to the ATP-binding site (e.g. Sorafenib); and type III, also known as 'covalent' inhibitors, which bind to Cysteine residues in various locations of Tyrosine kinases, establishing covalent bonds that are close to permanent (*Wu et al., 2014*). Allosteric inhibitors commonly fall into the category of type II inhibitors.

Allosteric drugs are an increasingly popular alternative to traditional drugs. This is because they commonly function as modulators rather than total agonists or antagonists, hence give a wider range of options in terms of, for instance, dosage. They also potentially act as more specific drugs, since the allosteric site or sites of protein are usually not as conserved as the more biologically relevant orthosteric sites (*Smyth and Collins, 2009*). For protein kinases, this is an especially important factor: although there are over 20 kinase families with their subsequent subgroups and are encoded by over 500 different genes in the human genome, the active site binding ATP is highly conserved, with a high sequential similarity across all characterised kinases, which poses a great challenge for traditional drug-like molecules that target the active site exclusively.

There are currently several kinase inhibitors that exhibit an allosteric mode of action. The first kinase inhibitor, treatment of chronic myeloid leukaemia and inhibitor of Bcr-Abl, Imatinib (Gleevec), although it primarily binds to the orthosteric site of Bcr-Abl, also has been shown to bind to an allosteric site in the kinase domain (*Zhang et al., 2010*). Chronic Myeloid Leukemia is caused by mutation on the Philadelphia chromosome, origin of the fusion gene and protein Bcr-Abl. This fusion protein

prevents the natural auto-inhibition of c-Abl by the SH3 domain and the coverage of the myristoyl binding pocket, which also has been shown to have an auto-inhibitory function on the kinase (*Chico et al., 2010*).

In recent years a number of allosteric ligands and novel allosteric binding sites have been discovered in protein kinases. For example, p38 (also known as MAPK14 (Mitogen-activated protein kinase 14)). It is activated in response to stress stimuli, such as cytokines, UV irradiation, heat shock and osmotic shock. It is involved in cell differentiation, apoptosis and autophagy. This kinase is medically very attractive since its inhibition may have a therapeutic effect on autoimmune diseases and inflammatory processes (*Pargellis et al., 2002*). p38 has been recently discovered to have an allosteric pocket within its kinase domain, (*Swann et al., 2010*). A novel drug-like inhibitor has been successfully synthesised and X-ray crystallography structures have been obtained with the inhibitor binding to the allosteric site and ATP binding to the orthosteric site (*Fabian et al., 2005*). Other kinases with potential for allosteric therapies include the JAK kinases and PDK-1 (*Sadowsky et al., 2011*) (*Wan et al., 2013*).

Nevertheless, the deliberate strategy of searching for therapeutics with allosteric mode-of-action has been to date a minority pursuit in kinase research. Many allosteric ligands have been discovered to have allosteric binding to the target accidentally, and in some cases after a long time of being widely used. The main question this Masters project asks is: can we rationally detect the presence of active and targetable allosteric sites in protein kinases, and if so, can these be exploited for effective treatment of diseases in which these proteins are involved? This multidisciplinary project encompasses the use of bioinformatics for the rational detection of the allosteric pockets throughout the human kinome, and the application of a biophysical technique Surface Plasmon Resonance (SPR) for the screening of potential binders, and to ascertain their binding mode. SPR is a highly versatile technique widely used in drug discovery that provides a complete kinetic profile of the binding of a ligand to a target. With the use of instruments Biacore T200 for compound screen and assessment of protein activity and stability, and Biacore 4000 for fragment screening, allosteric sites in protein kinases can be successfully identified. The principle of SPR is an optical method to measure the refractive index on a sensor surface. The variation in the angle of the refractive index is given by the change in mass on the surface. This is later translated into Response units (RU) over time, giving as a result a sensorgram which is analysed and from which the kinetic properties of the analytes are extracted. In practice, the target analyte (in this case a protein) is captured on the surface of a chip, which consists of a gold layer with active carboxyl groups. The methods of

immobilisation differ: a protein can be captured via biotinylation and subsequently captured by a surface of streptavidin, it can also be done by the use of tags and antibodies and even by having Nickel in the surface and capturing via His-tag. Untagged proteins can be captured in any active amine groups on the carboxylated surface. This method is viable, although the lack of control of the position on which the target will be captured risks the shielding of active sites or any other inconvenient positions, leading to steric clashes and decreased efficiency on the experiment (*Tanious et al., 2008*).

The first protein that we are working with is Interleukin-2-induced T cell kinase (ITK). ITK is a tyrosine kinase belonging to the TEC family; involved in the development of hematopoietic cells, such as T cells, mast cells and NK cells (*Berg et al., 2005*)(*Qi et al., 2012*). ITK is a downstream activator of PLC- γ 1 (*Min et al., 2009*), which in turn regulates the activation of cytokines, hence having various effects in the immune system. This kinase is a validated target for autoimmune and allergic inflammatory diseases.

ITK has proven to have active allosteric sites in its kinase domain (*Han et al., 2014*), which are well-defined and targetable. The second protein is Src kinase, a member of the Src family kinases (SFK) which is also a tyrosine kinase. It is involved in a myriad of cancers, highly present in tumours, hence having an attractive potential for cancer therapeutics. These two proteins are part of a larger family of tyrosine kinases which have important implications in cancer and autoimmune disorders (*Bradshaw et al., 2010*) (*Harling et al., 2013*), hence they are the focus on this project.

The aim of this MSc project was to develop an efficient assay in SPR for the testing of dual site inhibitors in Tyrosine kinases, and to rationally explore the allosteric sites via computational methods.

1.2. Kinase importance for drug discovery

There are over 500 genes encoding for protein kinases in the human genome (*Manning et al., 2002*). Most aberrations in kinase function result in severe disruptions of cell function, since most of them are involved in cellular signalling. The pathways in which they are involved range greatly, from correct signal transduction in the synapses, to metabolism, to immune response, to DNA synthesis (*Forýtková et al., 1995*). The importance of keeping a careful control over protein kinases is therefore,

crucial. Nevertheless, many diseases are involved with deregulation or aberrations in protein kinases, especially in cancerous diseases.

The first kinase inhibitor that was FDA-approved and got significant success in disease management and treatment was Imatinib, or Gleevec (*Agrawal et al., 2010*) as it was marketed, and it is a kinase inhibitor of the aberrant kinase dimer Bcr-Abl, causative agent of Chronic Myeloid Leukaemia. This inhibitor is highly selective for Bcr-Abl, which is a hybrid of the kinase Abl and the aberrant protein Bcr, given by a genetic fault.

After the discovery of oncogenes in the 1970's protein kinases have attracted high levels of attention for potential targets for cancer therapy (*Pierotti et al., 2003*), and was further encouraged by the success of Imatinib. This resulted in an increased body of research in kinase drug discovery. Not long after, other therapeutics, including antibody-based kinase inhibitors for proteins such as Trastuzumab (*Lu et al., 2010*) which target epidermal growth factor receptor in breast cancer, gained popularity. Nonetheless, the difficulty with selectively targeting one protein instead of its whole family became evident. The orthosteric ATP-binding active site is challenging to be effectively targeted by traditional drugs since the ATP-binding pocket of protein kinases is highly structurally conserved, selectivity is a great issue (*Karaman et al., 2008*) when designing therapeutics for cancer. The potential side effects may be numerous and highly affecting the patient's quality of life, and it is difficult to select aberrant proteins from the unaffected ones, often within the same family despite their functions being often very diverse from one another.

An alternative to this issue is the use of allosteric drugs. These compounds target pockets other than the orthosteric site, which has several advantages: these pockets are not as well conserved as the ATP-binding site; they can be conformation-specific, meaning the therapeutics would only bind to a specific activity state of the protein; and they can single out unaffected kinases (*Comess et al., 2011*).

Protein kinases are often 'unlocked' from their inactive state by another protein kinase. When a protein is phosphorylated by an upstream kinase, the structure of the protein undergoes a conformational change that results in the exposure of the ATP-binding site to the solvent, then proceeding to recruit the corresponding substrate (*Bornot et al., 2013*)(*Engel et al., 2006*). Often, tumours occur due to a disproportionate activity of protein kinases resulting in increased cell proliferation and differentiation, as well as DNA synthesis and recruitment of receptors to specific places in the cell (*Davies et al. 2000*).

For these reasons, allosteric modulators have garnered significant attention in the last years, resulting in several therapeutics with this mode of action. Most allosteric drugs in the currently being researched and in clinical trials are bitopic ligands (*Wenthur et al., 2015*), which involves the binding of the compound to both the orthosteric and the allosteric site. This suggests that most allosteric pockets become available once the kinase in question is active. The possibility of binding to an inactive conformation of the protein is also attractive, but has been scarcely researched.

1.3. SPR: physical principles and applications

Surface Plasmon resonance is a biophysical technique that uses the physical phenomena of Plasmon resonance to detect and characterize kinetic interactions between an immobilised target and a ligand. It is a highly versatile technique that allows real-time identification of kinetic interactions that were previously very difficult to ascertain.

The main principle behind the optical technique SPR is the phenomenon that occurs when light is reflected from dielectric metals. At a given angle, the incident light reacts with the delocalised electrons on the metal film, which are called plasmons, due to the coupling of the incident photons to the surface's electrons, which then move as a single entity, giving place to an electromagnetic field. This angle is the one in which the wavelength of the incident photons coincides with the resonance wavelength of the metal that composes the film (*Tanious et al., 2008*). (*Crescenzo et al., 2008*). The most commonly used metal for SPR experiments is gold due to its stable and ordered atomic arrangement when distributed into a film, although silver can also be used. When a molecule of significant mass binds to the surface, it can alter the angle of the reflected light, which gives the relationship between mass and wavelength that confers SPR experiments their sensitivity (*Gauglitz, 2005*). This optical phenomenon can be utilised to measure interactions of ligands and targets on the surface of said metal layer. When a ligand is immobilised on the surface of the metal, with the calculated light reflecting at the specific angle, it is possible to measure interactions by the shift of the reflected light (*van der Merwe, 2001*). When the immobilised ligand binds to another body, which can be another protein, a chemical compound or a fragment, the reflected angle is shifted due to the mass variation in the surface, and can be detected by a detector (*Figure 1*).

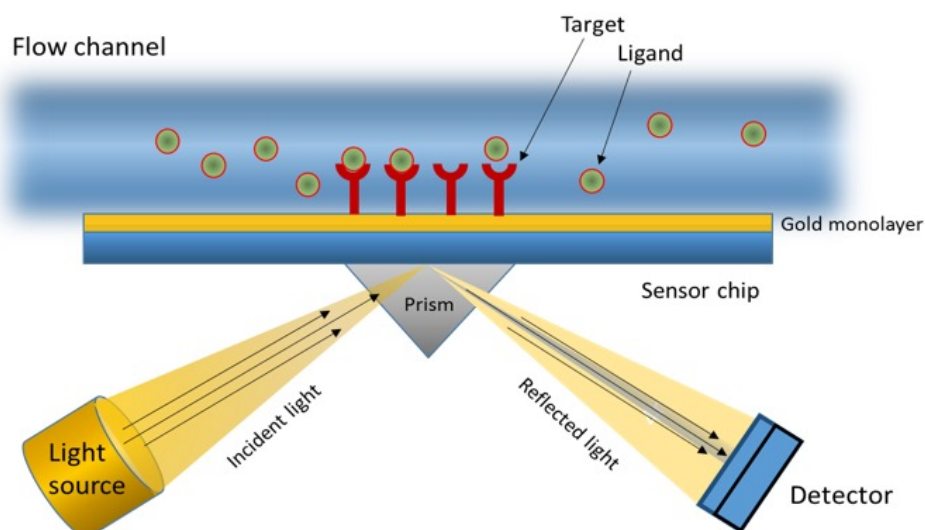


Figure 1: Diagram depicting the basic conformation of an SPR sensor. The sensor chip is coated with a dielectric metal, in most cases gold (as shown above). Light is incident on the back of the sensor chip where a prism is located. The prism allows for the reflection of this light, which is detected by an optical detector (blue). The angle is varied when the mass of the immobilised target (red) is varied when binding to a ligand (green) occurs. This shift is then converted to a curve that reflects the response units obtained, representative of the strength and kinetics of the binding.

Surface plasmon resonance (SPR) is a widely used biophysical technique in drug discovery. This can be used to measure interactions of objects on the surface, in this case, of binding of ligands to proteins immobilised on the surface. Specifically, SPR allows the analysis of the kinetic properties of the interactions of the compounds, where the protein is called the ligand or target, and the compounds are called analytes. The method can also be used to explore protein-protein interactions, nanobodies, fragment screening with a semi-high throughput outcome, and analysis of protein stability. Directly the association and dissociation rates of the kinetics (k_d , k_a) and thus the affinity (K_D) (Hahnefeld *et al.*, 2004) are given. SPR assays do not require a high concentration of target protein, nor do they necessarily require a high purity (depending on capture method). Thus these properties makes a very favourable technique to utilise in otherwise challenging assays where the quantities of high purity required are very high, such as in NMR, or where the protein inherent properties are against the technique's requirements, as it is with the case of X-ray crystallography for membrane proteins, for instance.

SPR is a very versatile technique, which can offer enlightening results on protein behaviour, stoichiometry, binding mode and kinetics of the interaction. It is unique in its low demands of protein purity and quantity, and can be utilised to measure protein complexes interaction with ligands, small molecules, drug molecules, and other

proteins and macromolecules, such as nanobodies and phospholipids (*Aristotelous et al., 2013*). A key advantage found in SPR is the label-free nature of the protein, and the fact that it can identify ligand binding with high sensitivity with recent advances, makes it a highly desirable technique in early stages of the drug discovery process.

1.4. Fragment screening in protein kinases

Fragment-based drug discovery involves the screening of low molecule weight molecules (less than 300Da). Fragments are often based on existing drug compounds that have been broken down into component substructures, known as fragments. Fragments are compiled into libraries that can be used to run high-throughput screening. The aim of fragment based drug discovery is to screen small molecules that, albeit weak binding, can establish a high-quality interaction with a target, and can then be further optimised using the fragment's structure as a template to obtain a full-sized drug-like molecule (*Murray et al., 2012*)(*Lu et al., 2013*). Fragment based drug discovery (FBDD) can be implemented in a variety of techniques, including NMR to elucidate Structure-activity relationships (SAR) studies, X-ray crystallography being the main screening platform for structure drug design, and biophysical and optical methods, including SPR and Octet technologies. Less commonly utilised techniques in fragment screening include Mass Spectrometry, which is emerging as an attractive alternative to more traditional HTS and FBDD techniques.

FBDD is broadly applicable to a wide range of recombinant, soluble proteins, including proteins such as kinases (*Scott et al., 2013*). In FBDD a protein is screened against a fragment library, and SAR studies that can establish key interactions within the target's active site to elaborate effective lead compounds. FBDD is comprised of three stages: design of fragment library, fragment screening, and lead elaboration and optimisation (*Mashalidis et al., 2013*). Stage two consists of biophysical techniques in which the fragment library is screened in vitro against the target. The most commonly used techniques for this are SPR, NMR, Thermal Shift and in some cases Mass spectrometry.

The versatile NMR technique can be used for FBDD. The requirements of protein NMR nonetheless, prove difficult to fulfil due to the large quantity of highly pure protein needed (*Ciulli & Abell, 2007*). There are several NMR variations used in FBDD, such as Ligand-detected 1D ¹H NMR and WaterLOGSY, a 2D NMR technique.

Thermal shift or differential scanning fluorimetry is a fluorescence-based technique that measures the thermal stability of a protein which when bound to a compound experiences an increase in melting temperature. This is a useful technique to identify ligands that successfully induce a conformational change in a protein that is subsequently represented by a thermal shift in the melting temperature of the protein, and can be detected by means of sensitive fluorescent dyes (*Silverstre et al., 2013*). This technique does not provide, nonetheless, highly reproducible data, which is why it is often used as a support technique for fragment screening, and not the main one (*Silva-Santisteban et al., 2013*).

X-Ray crystallography is a highly treasured technique due to the validity of the outcome: it can validate structural binding of the ligand to the protein, provide information of the folding and the three-dimensional structure of pockets, which can help further the lead optimisation of drug-like molecules. For FBDD, target crystals are incubated with individual fragments of typically 50nM concentration (*Murray et al., 2012*), or with high-concentrations of a “cocktail” of fragments. The difficulty in this technique lies primarily with the fact that obtaining crystals of proteins is highly complex especially in the case of membrane proteins (*Velvedapu et al., 2015*)(*Patel et al., 2014*) In the case of kinases crystal structures are often only available for only individual domains and Apo structures are scarce (*Erlanson et al., 2011*).

Surface Plasmon resonance is a biophysical alternative to traditional High Throughput Screening techniques, and the rest of the techniques used in FBDD. It is a highly versatile technique that primarily consists of immobilising a target onto a metal surface and injecting a series of fragments through it. The binders are detected by the mass variation on the surface, causing a shift in the reflected angle on the detector, and the binding kinetics of the association and dissociation of ligand-target are obtained. Due to recent advances in technology development, SPR assays may screen hundreds to thousands of fragments in a short period of time, offering a close to high-throughput screening result. This is the primary technique utilised in this Masters project.

1.4.1. Src kinase as a drug target

Src kinase is encoded by the Src kinase proto-oncogene in the human genome. Src kinase belongs to the Src family of protein tyrosine kinases (SFK) along with kinases Yes and Fyn and its role is related to many essential aspects of cellular life and survival, involving cell growth, shape, and differentiation, as well as specialised cellular signalling (*Parsons & Parsons, 2004*). Src kinase is constituted by various domains:

the kinase domain where the ATP-binding site is located, the SH2 and SH3 regions, which act as auto-inhibitory domains and involve most of the interactions with other proteins and the tail region (*Ottenhoff-Kalff et al., 1992*), a short regulatory domain. As with many other protein kinases, there is an interest in Src kinase as a potential drug target for cancer therapeutics, since it has been shown that Src kinase aberrations are encountered in a high percentage of tumours.

SFKs are activated downstream of many receptor tyrosine kinases, including Platelet-derived growth factor (PDGF), the epidermal growth factor receptor (EGF-R) and insulin-like growth factor-1 receptor (IGF1R) (*Bromann et al., 2004*). The kinases of the Src Kinase family interact with the RTKs via their SH2 domain, which contains an active Tyrosine residue that becomes phosphorylated in response to interaction with the receptors, and it provokes the unlocking of the protein which renders it catalytically active (*Roskoski, 2005*), changing the conformation of the protein to reveal the ATP-binding site, where the protein utilises ATP to phosphorylate Tyrosines on the downstream substrates. Downstream effects of Src kinase activation include mitogenesis, angiogenesis. Src kinase is known to closely interact with and regulate important factors in cellular signalling, including the RAS pathway and the internalisation of EGFR internalisation (*Ware et al., 1997*). It also correlates with higher levels of VEGFR and Angiopoietin-2 (*Zan et al., 2011*) in rat brains with cerebral ischemic injury.

Levels of Src kinase have been observed to be higher in several cancers, including colon, breast and lung cancer (*Sirvent et al., 2010*) (*Kopetz, 2007*). Due to genetic redundancy (*Thomas et al., 2002*), an exact role for Src kinase is yet to be determined. Nevertheless, it has been experimentally proven that uncontrolled activity of Src kinase results in rapid growth and differentiation of cancerous cells (*Shukla et al., 2013*). Inhibition of Src kinase activity by multi-kinase inhibitors such as Saracatinib has proven to alleviate symptoms in chronic myeloid leukaemia, and is currently being tried in lung carcinoma and breast cancer (*Bitencourt et al., 2011*).

Hence, the stabilisation of the inactive conformation of Src kinase via allosteric inhibitors is an attractive alternative for both therapeutic ends and further discovery of the mechanism of action of this protein.

1.4.2. ITK as a drug target

Interleukin-2-inducible T-cell kinase (ITK) is a non-receptor tyrosine kinase encoded in the ITK gene in the human genome. It is a member of the TEC family of tyrosine kinases, and it consists of several domains: the kinase domain, where the active ATP-binding site is located, a Pleckstrin-homology (PH) domain, an SH2 domain and an SH3 domain and a TH domain. ITK plays an important role in adaptive immune response of antigens, since it is involved in T-cell differentiation and proliferation (*Sahu & August, 2009*). ITK is a positive regulator of T-cell signalling. ITK is phosphorylated, along with the rest of the TEC family, in response to T-cell receptor (TCR) activation. It is an important factor in T-helper cell differentiation into either Th-1 cells or Th-2 cells (*Biedermann et al., 2004*). Th-1 and Th-2 cells have phenotypically different functions: Th-1 cells act against the host's intracellular bacterial and protozoan organisms, whereas Th-2 cells are mainly effectors against extracellular parasitical organisms.

On activation of the T cell receptor, PI-3 kinase is activated and consequently phosphorylates Phosphatidyl inositol 3, creating the substrate for the PH domain of ITK to bind. This triggers the recruitment of ITK and the formation of dimers with receptors that also activate PI-3 kinase. Once ITK reaches the cell membrane, it is phosphorylated by protein kinases of the Src kinase family, closely related to the TEC family (*Berg et al., 2005*). This phosphorylation activates ITK to interact with its SH2, SH3 and TH domains, which leads to the downstream activation of important signalling components such as CY1 and Calcium²⁺ (*Wilcox & Berg, 2003*). ITK also signals through NFAT (*Brown et al., 2004*) for the transcription of several cytokines associated with inflammatory responses. ITK is therefore an attractive target for therapeutic intervention in autoimmune and allergic diseases. ITK^{-/-} deficient mice have decreased responses to allergens, especially those attributed to asthmatic responses (*Qi et al., 2012*), which make it an attractive alternative for treating severe asthma, instead of the symptomatic treatment currently offered by broncho-dilators such as Salbutamol and its steroidal counterparts (*Bello & Njoku, 2007*) and the less-than-ideal alternative of anti-histamine treatment. ITK is especially inviting as a target because its action seems to be more restricted to Th-2 cells, which would signify reduced side effects and more effective treatment for overactive Th-2 cells present in the lungs (*Sahu et al., 2008*).

1.4.3. ITK as an allosteric drug target

In recent research conducted by Pfizer (*Han et al., 2014*), ITK has been shown to possess allosteric pockets that are highly druggable. One publication obtained an efficient compound that functions as a bitopic ligand, which is a ligand that simultaneously binds to the orthosteric site and an allosteric one, possibly by the activation of the orthosteric one and subsequently the alternative site becomes occupied (*Kamal and Jockers, 2009*). A crystal structure was obtained, and biophysical assays were performed. On this basis, SPR experimental studies were conducted to confirm the allosteric activity of the discovered inhibitor, and the effectiveness of the binding to the pocket present in ITK and perform fragment screening studies to identify possible fragments with an allosteric mode of action. A library of over 600 fragments courtesy of the University of Dundee's Drug Discovery Unit (DDU) was employed in this study, due to structural similarities to the published allosteric inhibitor.

2. Methods Section

2.1. Computational studies

As a first step, we took the crystal structure of ITK published by Han et al, 2014 available on the Protein Data Bank (PDB, identifier 4M0Y) and analysed the allosteric site in PyMol. This structure consisted of the kinase domain of ITK bound to the allosteric inhibitor described in this publication. The ligand was bound to two sites, the allosteric site and the orthosteric one. The residues of both pockets were mapped and extracted, and the interactions with the allosteric inhibitor were defined and isolated, as seen in Figures 2 and 3.

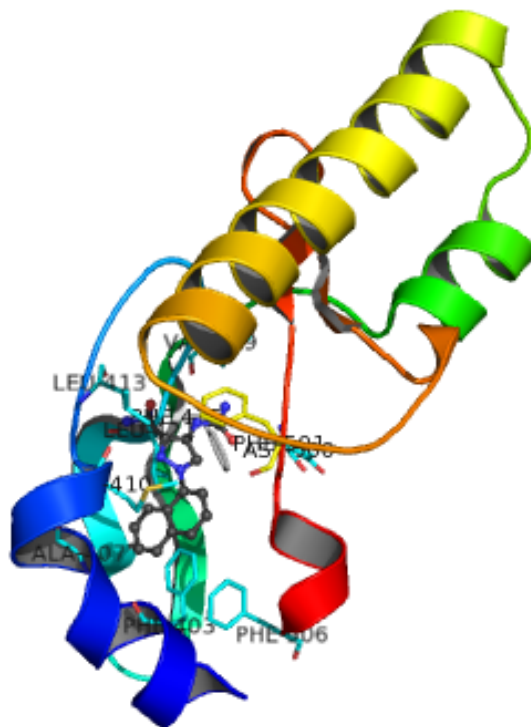


Figure 2: *View of the allosteric site with highlighted residues directly interacting with the allosteric inhibitor.*

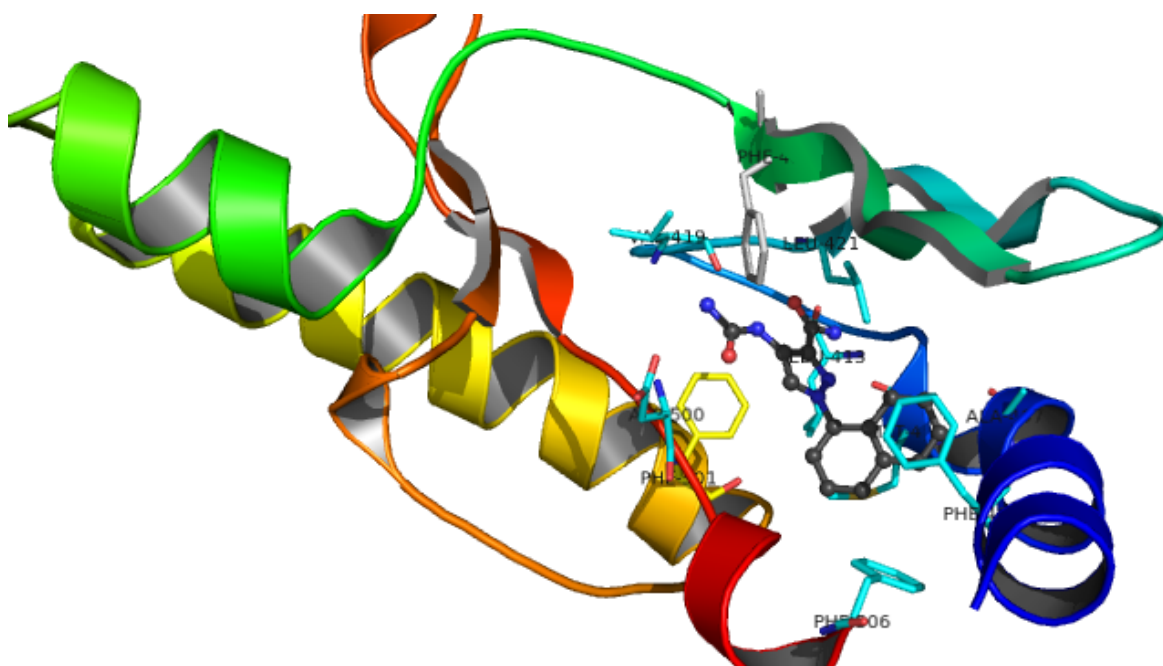


Figure 3: *Cartoon representation of the allosteric pocket of ITK, part of the kinase domain whose sequence spans from residue 399 to 506.*

From the crystal structure, the residues taking part in the allosteric binding site with interactions to the inhibitor were identified. A BLAST search was performed in UniProt database, and the residues identified were highlighted as follows:

399 – S E E D F I E E A E V M M K L S H P K L V Q L Y G V C L E Q A P I C L V F E
F M E H G C L S D Y L R T Q R G L F A A E T L L G M C L D V C E G M A Y L E E
A C V I H R D L A A R N C L V G E N Q V I K V S D F G M T R F – 506

As shown in the sequence above, the allosteric pocket is formed by sparse residues. Following this identification, a preliminary sequential analysis of ITK plus the rest of the TEC family kinases (BTK, TEC, TXK and BMX) was performed using Jalview 2 desktop application. The kinase domain was color-coded by similarity using a BLOSUM62 matrix. The residues were then color-coded using ZAPPO colour scheme (figure 4), in which the physico-chemical properties of the amino acids are represented in different colours. The overall consensus shown when these sequences were aligned was significant. As expected, the TEC family of kinases share a remarkable similarity in their kinase domain, and also being practically identical in the residues that form the allosteric pocket in ITK excepting for Phenylalanine 435, which is exclusive for ITK. The following tyrosine kinases were analysed: from the TEC family: ITK, TEC, TXK, BTK and BMX; Abl1 and Abl2; FES, FER, FRK, BLK, SRC, FGFR1, VEGF1, EGFR1, ERBB2; and finally the three members of the Janus kinase family. P38 was also added to this alignment in order to assess whether its BIRB-binding site would correspond to the one reported to ITK. The residues in p38 and ITK allosteric sites are largely similar physico-chemically, although the residues binding to their inhibitors are different.

Aliphatic/hydrophobic	ILVAM
Aromatic	FWY
Positive	KRH
Negative	DE
Hydrophilic	STNQ
Conformationally special	PG
Cysteine	C

Figure 4: *Zappo colour scheme. This scheme is particularly useful since it codes the residues by their physico-chemical properties.*

The allosteric site in p38 is in the opposite side of the molecule instead of by the orthosteric binding site, contrasting with ITK. Nevertheless, this may suggest that despite the conformation, the residues form the allosteric pocket independently.

2.2. ITK: protein production and SPR assays

2.2.1 Protein Production

The cloning, expression and purification of the protein was done in the Structural Genomics Consortium in Oxford, as part of a collaboration project. The ITK construct containing the kinase domain and the SH2 domain was successfully cloned, expressed and purified at the SGC Oxford facilities. The purification was carried out via Nickel affinity column, and SDS-PAGE gels were taken at each step. Final purification was performed via Gel Filtration. Mass spectrometry TOF was carried out to assess purity and phosphorylation states of the protein. The results of this are expressed as follows.

2.2.2 Construct design

Staff at the Structural Genomics Consortium (SGC) in Oxford designed and cloned the construct of ITK. The protein expressed is a C-terminal 6His-tagged construct containing the kinase domain and the SH-2 domain of ITK. This is wild type, free of included mutations. The construct in the expression vector was obtained from GenScript™.

2.2.3. Protein expression

Cloning and amplification of the construct was performed by staff at SGC Oxford. P2 generation of virus was used to infect Sf9 cells. Insect X-press with L-Glutamine (LONZA) was used for culturing insect cells, with a ratio of 1:4 cells to media (300mL of cells to 500mL of media). Penstrep Streptomycin (1mL/L) was added to the culture. Virus was inoculated into the media: 1mL/L. Immediately after infection, cultured cell mixture was transferred to 2L conical flasks and put to incubation in the following conditions: 27 C at 90rpm for 72h. Total volume was 6 x 800mL, approximately to yield 2 million cells. Resulting infected cells were harvested and centrifuged at 900G (~1900rpm, 4°C, for 20min). Supernatants were centrifuged and the resulting pellets

conserved. All pellets were re-suspended using PBS buffer and centrifuged once again, before freezing at -80°C for 24h.

2.2.4. Purification

The following buffers were prepared for purification: 1L of 2M Imidazole stock at pH 7.5 for elution, and lysis buffer consisting of 2L of 50mM HEPES, 0.5M NaCl, 5% glycerol, 1mL/L and 0.5mM TCEP at pH 7.4. The cell pellets were thawed while kept on ice, and sonicated for 2min in cycles of 10s at 10Amp. DNA was precipitated using PEI (0.5% of 5% stock) and pellets were centrifuged at 15500rpm for 1h at 4°C. Small volume of total was preserved for analysis in SDS PAGE.

2.2.4.1. First Nickel column

A Nickel column was set up. Washes were done three times with MilliQ water and with Lysis buffer. 10mL of Nickel slurry was loaded onto the column. Supernatant of cells was flowed through the column and collected. The column was washed twice with different ratios of salt to imidazole: first 50mL water, 20mM Imidazole and 0.5 NaCl; second 50mL water and 20mM Imidazole. The elution of the protein was achieved by loading the column with 50mM of 300mM NaCl solution. An SDS PAGE gel including the total, flow through, the two washes and the elution was set up. Following SDS PAGE results, cleavage of the 6His tag was achieved by dialysis with TEV protease (1:25 ratio) for rebinding to Ni column and further purification. Dialysis buffer consisted of: 50mM HEPES, 300mM NaCl, 0.5M TCEP, 5mM MgCl₂ at pH 7.4. The dialysis was carried out at 4°C overnight, with the use of SnakeSkin™ dialysis film, under soft stirring.

2.2.4.2. Rebinding of Nickel column

Fresh buffer (150mM NaCl, 20mM HEPES, 5mM MgCl₂, 0.5mM TCEP at pH 7.4) was prepared, in addition to an Imidazole concentration gradient in the aforementioned buffer (10-50mM in 10 increments, and 250mM). The dialysed protein was loaded onto the Nickel column and the flow through was collected. Subsequently, the imidazole gradient was added to the column and collected. An SDS PAGE gel of each step was performed.

4.1.4.3. Gel Filtration

As the final purification step, a Gel filtration column was performed in an AKTA system. For this, the approximate resulting volume of 80mL was concentrated into 6.5mL via

three centrifugation cycles at 3900rpm for 15min in spin columns. The gel filtration column was loaded in the AKTA and the procedure was started overnight at 4°C.

2.2.5. Mass spectrometry

Experimental analysis with Agilent TOF mass spectrometry was carried out by staff at the SGC Oxford. Using NanoDrop technologies, the average concentration of protein was found to be 2.55mg/mL.

2.3. ITK SPR assay development and fragment screening

Surface plasmon resonance was the technique utilised to assess protein activity, binding kinetics, and fragment based screening studies. The instruments used were mostly Biacore T200 and Biacore 4000 for the fragment screens for ITK. Sierra Technologies MASS-1 instrument was also employed in experimental setup and assay development, as well as fragment screen and confirmation studies of another kinase in this study, Src kinase.

Running buffer modifications were implemented to optimise the binding activity of ITK. Several experiments were introduced: immobilisation of SA on CM5 chip versus capture with a SA coated chip from GE technologies; addition and elimination of Magnesium chloride in the running buffer. The magnesium has been seen to aid in protein activity performance in biophysical assays (*Lovitt et al., 2010*). As an attempt to preserve biological conditions as much as possible, an assay without manganese in the running buffer was performed. Firstly, the activity of ITK was assessed with a basic binding assay.

2.3.1. Validation of protein activity

2.3.1.1. Preconditioning and surface activation

Preconditioning of the surface was performed following standard Biacore procedures. On a CM5 chip and the instrument primed to HBSN (10mM Hepes, 150mM NaCl, pH 7.4) at 25°C of temperature, the surface was preconditioned by injecting 100mM HCl, 50mM NaOH, 0.5% SDS and MilliQ water at a flow rate of 100uL/min and 10s contact time. Surface activation was performed by mixing 1:1 ratio of EDC and NHS solutions (750 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 115 mg N-hydroxysuccinimide (NHS), Biacore).

2.3.1.2. Streptavidin immobilisation: preconcentration, immobilisation and deactivation

The first experiment performed on ITK was done following a published protocol for this assay (*Han et al., 2014*).

Preconcentration of streptavidin was performed using Acetate solutions (Biacore) of pH 4.0, 4.5 and 5.0 at 25°C and under HBSN running buffer. A volume of Streptavidin was mixed in 200uL of each acetate solution and injected over the surface. The optimal pH for SA was found to be pH 4.5 and it was used from this point onwards. Streptavidin was dissolved in Acetate solution pH 4.5 and immobilised via amine coupling on the surface (flow cells 3 and 4). Once immobilised, the surface was deactivated with Ethanolamine solution (1.0 M ethanolamine-HCl pH 8.5, Biacore) injected for 4 minutes.

2.3.1 3 ITK biotinylation, capture and assay

ITK was biotinylated and captured onto immobilised SA surface on CM5 chip in Biacore T200.

A 1:1 ratio of biotin to protein was employed to biotinylate ITK. The biotin used was EZ-linked NHS biotin from Thermo-Fischer Scientific. The biotin was added to the thawed protein aliquot and incubated on ice for 2h. The biotinylated protein was desalted using desalting columns (company information, porosity) by centrifugation cycles of 2000rpm at 4°C for 1min at a time. The instrument was set at 4°C assay temperature and primed to the assay buffer (50mM Tris-HCl, 150mM NaCl, 5mM MgCl₂, 1mM DTT, 1mM MgCl₂ and 0.05% Tween20 at 3% DMSO) and captured onto one flow cell. The remaining flow cell with the immobilised SA functions as the reference flow cell for the experiment. This protocol is the standard immobilisation/capture protocol followed for ITK assay studies, and will be referred to for following assays.

For validation of target activity, an assay was set up with concentration series of Adenosine triphosphate, the protein's natural ligand, and a commercially available inhibitor with known allosteric activity synthetised by the European Screening Centre (Newhouse, Lanarkshire). The assay was setup for two-fold dilutions of ATP at six concentrations, with starting concentration being 500 µM. For the Pfizer inhibitor, the assay consisted on three-fold dilutions at 6 concentrations with a starting concentration of 10 µM. The DMSO concentration was adjusted for both analytes to match the 3% DMSO present in the running buffer and at 4°C temperature.

2.3.2. Assay development

2.3.2.1. Optimisation of protein activity

Running buffer modifications were implemented to optimise the binding activity of ITK. Several experiments were introduced: capture with a SA coated chip from GE technologies,

2.3.2.2. Assessment of Manganese influence in assay

Following the previously described protocol, a Biacore CM5 chip was docked and the system was primed with HBSN. The surface was preconditioned at a temperature of 25°C. The surface was activated for NA immobilisation with a 1:1 mix of NHS and EDC on both flow cells 1 and 2. NA was immobilised using a concentration of 5mg/mL in pH 5 acetate solution. The end immobilisation response was of 7000RU. The surface was then deactivated using Ethanolamine. The system temperature was decreased to 4°C for capture of ITK. Once temperature was decreased, T200 was primed with running buffer. Biotinylated ITK was captured onto the surface and an assay (same as previous experiment) was run.

2.3.2.3. Validation of allosteric activity

Competition studies with an orthosteric ITK inhibitor purchased from Merck (*Lin et al., 2004*) were performed. The binding of this inhibitor to ITK was tested with an activity assay consisting of serial dilutions of the compound and injected on an ITK surface as previously described. After confirming the binding of this compound, a competition assay was performed in which 1µM of Merck inhibitor was present in the running buffer. The assay plate consisted of injections of serial dilutions of ATP at 200µM and the allosteric Pfizer compound at 10µM. The assay conditions remained the same as previous assays. The allosteric activity of the Pfizer compound would be confirmed if binding exists in the presence of constant blocking of the slow off-rate inhibitor.

2.3.2.4. Protein stability over time: Z-Prime

The next step in the assay development for the target ITK is to assess the stability and activity of said protein over time, with an experiment of a similar length as the fragment screen: 48h.

ITK was captured on streptavidin surface on flow cells 1 and 3, leaving flow cells 2 and 4 as reference. Flow cell 3 was blocked with slow off-rate inhibitor BMS. ATP was

injected at 200 μ M over all flow cells in 1 hour intervals over 48 hours and at concentration series 6.25-200 μ M at the beginning and end of the screen. The purpose of these concentration series was to monitor the binding ability of ITK at the beginning and the end of the experiment, over all flow cells. Expected to see was that the binding in fc1 would be superior to that in fc3, since this flow cell would be blocked by the the Merck orthosteric inhibitor.

2.3.3. Fragment screening

A fragment library of 656 fragments obtained from DDU (reference) was screened against ITK on Biacore 4000.

The assay was carried out on a Biacore Streptavidin chip at 4°C. ITK was biotinylated as described and was captured on spots 1 and 5 on all flow cells. On this instrument, it is recommended that spot 3 is always left as a reference. The surface was blocked with unlinked biotin after the capture.

Concentration series of ATP (6.25-200 μ M) were injected at the beginning and end of the experiment as activity controls. A concentration of 10 μ M of the Merck orthosteric compound was injected on spot 5 to block the surface. This was to identify fragments with possible allosteric activity. Fragments were screened at 3 concentrations: 150, 50 and 16.6 μ M.

2.3.4. Hit confirmation

The fragments that resulted in a positive binding to ITK were selected from the source plate and diluted to a 150 μ M concentration. This assay was performed in Biacore T200 due to its increased sensitivity and ability to detect the kinetic profile of the fragments. The assay setup was the same as for the fragment screen. The confirmation was done twice: the first time with normal running buffer, and the second time with 10 μ M of the orthosteric inhibitor present in the running buffer for identification of fragments with allosteric binding.

2.4 Src kinase Fragment screening

2.4.1. Protein production

Full length human Src kinase was cloned, expressed and purified by The Division of Signal Transduction Therapy, University of Dundee, and purchased for this thesis. The protein is N-terminally tagged with 6His tag. It was cloned and expressed in

baculovirus expression vector system. It was purified by Nickel 2+ NTA agarose column. The expression levels were 5mg/mL, and the purity achieved was of approximately 85%. The protein was stored in Tris buffer (50mM Tris-HCl, pH 7.5, 270mM Sucrose, 150mM NaCl, 0.1mM EGTA, 0.1% 2-mercaptoethanol, 0.02% Brij-35, 0.2mM PMSF, 1mM Benzamidine) at -70°C.

2.4.2. SPR assay development

The SPR experiments for this target was performed on a MASS-1 instrument (Sierra Sensors).

2.4.2.1 Validation of protein activity

Src kinase was captured by means of amine coupling to the surface of a CM5 chip in MASS-1. It was captured on Spot B of all flow cells, with Spot A functioning as a reference. Activity of the protein was tested using a concentration series of a published inhibitor (ChemBL 249097 compound).

The system was primed with HBS-P buffer (150mM NaCl, 10mM Hepes and 0.005% Tween20, pH 7.4) and set to 25°C temperature.

Preconditioning of the surface was performed using four solutions: 100mM HCl, 50mM NaOH, 0.5% SDS, and H₂O. The surface was then activated with a 1:1 ratio of NHS:EDC. The immobilisation of Src kinase was then performed. Src kinase was injected at 18 ug/ml in 10 mM Acetate pH 5.5 followed by injection of 1 M Ethanolamine.

To confirm activity of immobilised Src kinase, ChemBL249097 compound was injected at serial dilutions over Src kinase at concentrations 0.0044 – 1 µM in the running buffer 150mM NaCl, 50mM Tris-HCl, 5mM MgCl₂, 1mM DTT and 0.05% Tween20 at pH 7.5, 3% DMSO

2.4.2.2. SPR Fragment screening

A fragment screen was performed using DSTT's Src kinase. The experiment had as a goal to identify possible allosteric activity of the kinase. The fragment library used was obtained from the DDU (Drug Discovery Unit) of the University of Dundee, and consisted of 656 fragments.

The assay buffer used for the fragment screen was the same as the assay buffer for the activity test. Src kinase was immobilised on all flow cells on spot B, leaving spot A as a reference, and using a fragment library consisting of over 656 fragments.

Fragments were screened at concentration 50 μ M in running buffer 150mM NaCl, 50mM Tris-HCl, 5mM MgCl₂, 1mM DTT and 0.05% Tween20 at pH 7.5, 3% DMSO. ChEMBL compound was injected at 1 μ M during the screen to determine possible activity loss over time and also at concentration series 0.0044 – 1 μ M at the beginning and end of the screen.

Source plates of fragments were stored in 100% DMSO at a concentration of 50mM. The target assay concentration was 50 μ M at 3% DMSO.

Solutions for the control plate were prepared beforehand in glass vials and then transported to 96 deep well plate (DWP) (400 μ L) for subsequent transport to the designated wells in 384 DW assay plate (120 μ L). The control plate included the DMSO calibration curve, serial dilutions of the ChEMBL compound at the beginning and at the end as control, and several wells with 1 μ M concentration of the ChEMBL compound.

The fragment screen was performed for 8 fragment plates, four each experiment. Both experiments were identical in their assay buffer, and the second one was performed over the same surface as the first one.

2.4.2.3 Hit confirmation

A hit confirmation assay was performed in Biacore T200 with the binders obtained from the fragment screen. The conditions were the same as the screen in MASS-1, with the amine coupling on a CM5 chip. The concentrations of the fragments were 56pM to 41nM 3-fold concentration series. As control, 1 μ M of ChEMBL compound was injected after every 16th injection of sample. 1 μ M Dasatinib was injected for blocking and for competition studies with fragments. This determines possible allosteric binders.

3. Results

3.1 ITK protein production and purification

The expressed construct was 46kDa in mass. Presence of the band between 55 and 40kDa strongly suggested the presence of the construct. The first purification yielded a strong band in the region where the 6-His tagged protein was to be expected, with several bands suggesting impurities. The second Nickel column, which involved rebinding to the column to further the purification and cleavage of the His tag (figure 5), yielded promising results: little impurities were observed. The middle three bands, corresponding to imidazole gradients of 10, 20 and 30mM were the ones selected to undergo Gel filtration for the final purification step.

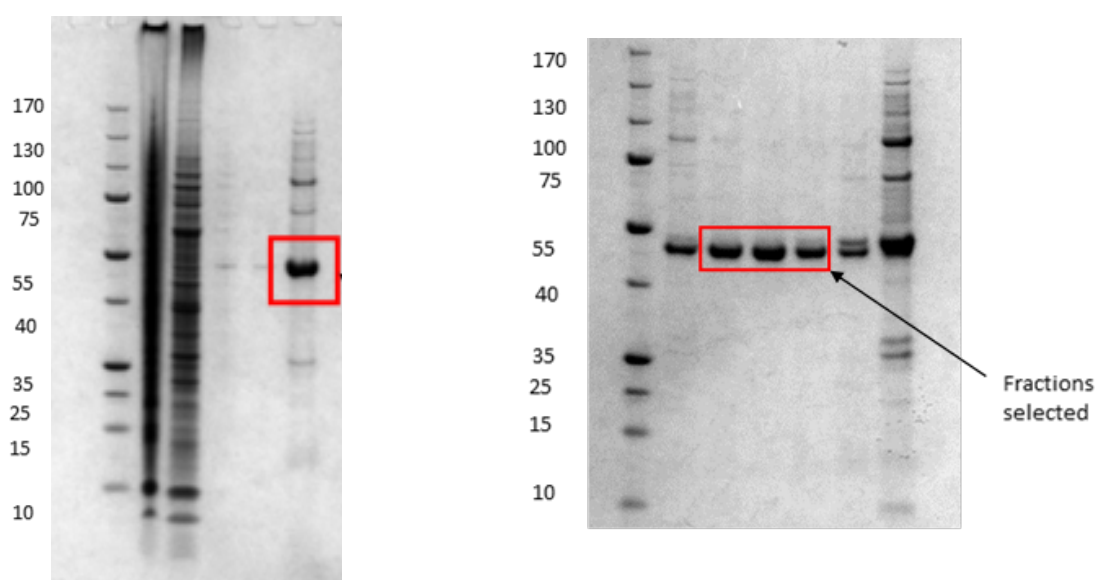


Figure 5: *First purification on the Nickel column (left). The band between 55 and 75kDa shows the presence of the expressed construct. Rebinding to Ni column (right). The fractions selected correspond to imidazole gradients.*

Gel filtration chromatography was performed by staff at the SGC (Figures 6 and 7). Five fractions were selected and the chromatogram was extracted for purity. These fractions were collected and concentrated for mass spectrometry experiments. The concentration of the finalised protein was assessed via NanoDrop, and was 2.55mg/mL.

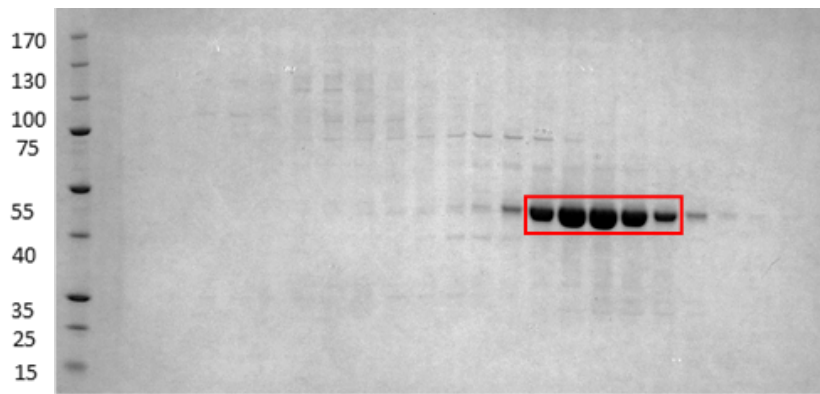


Figure 6: *SDS PAGE of gel filtration fractions. The fractions selected correspond to the peak in the chromatogram.*

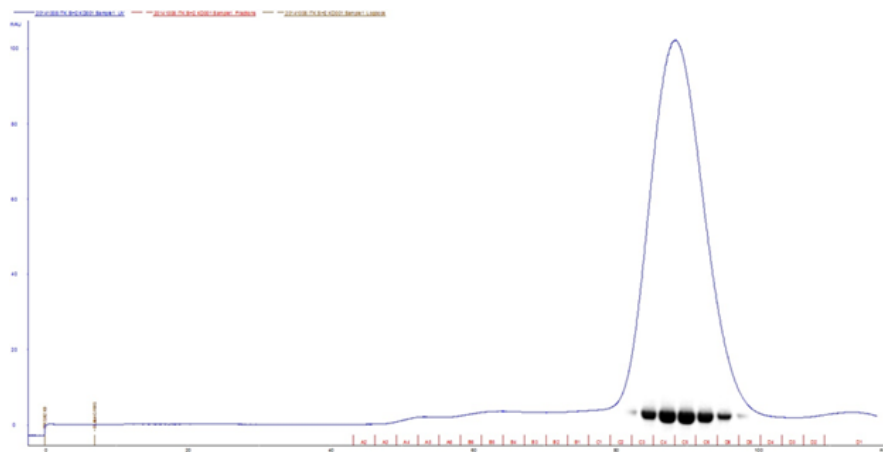


Figure 7: *Superimposition of the gel and the chromatogram illustrating the selected fractions.*

The analysis with Agilent TOF (Figure 8) revealed that the protein is partially phosphorylated. This is indicated by the presence of evenly-distanced peaks with accurate expected mass that are 80 or multiples of 80 apart. This is evidenced by the annotations in the analysis picture. The protein is believed to be approximately 55% phosphorylated, although this could only be known for sure via phosphor-mapping. *If there was a desire of phosphorylating or dephosphorylating the protein, there are ways to do so via phosphatases for instance.*

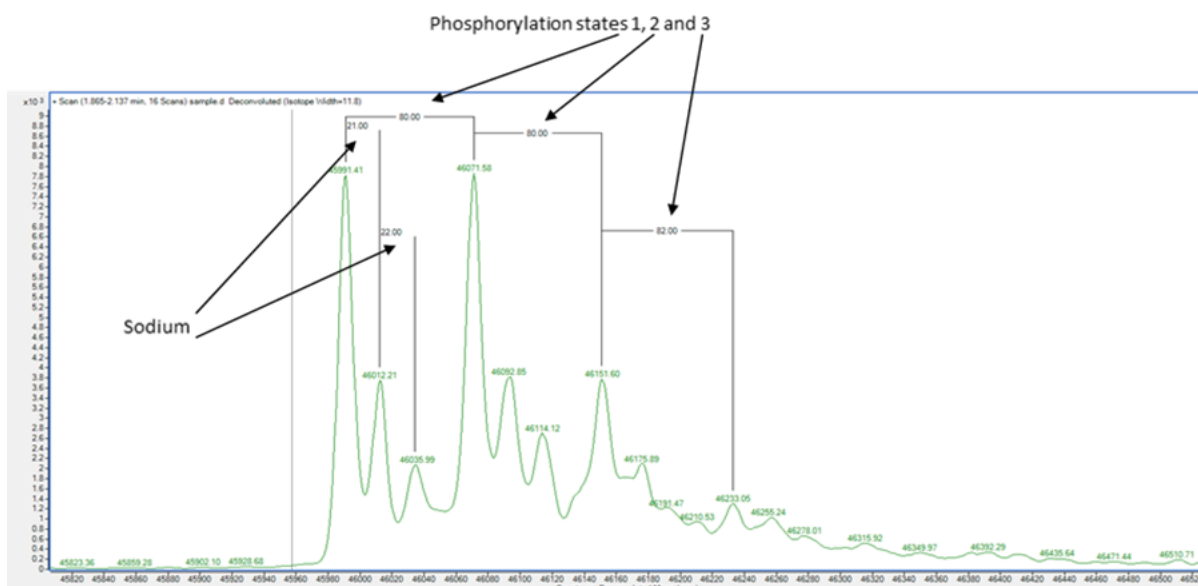


Figure 8: *Analysis of mass spectrum. The 21 and 22 annotations correspond to Sodium, and the 80 and 82 annotations correspond to phosphorylation states of the protein. To be considered phosphorylated, a protein ought to exhibit at least three of these peaks at a distance of 80 or multiples of 80.*

3.2 SPR Assays and Fragment screen

3.2.1. Activity test

The protein expressed and purified at SGC Oxford proved to be highly active. The protein was successfully biotinylated and the capture levels were adequate on each experiment, averaging the 3000-5000 RU (see Figures 9 and 10 for preconditioning and capture, respectively). The chemical biotinylation was effective and highly reproducible, resulting in active protein. The primary concern with chemical biotinylation is the 2h incubation on ice might result in a loss of activity on the protein after being thawed. Nonetheless, ITK proved to be a stable protein that could retain activity even after 2 cycles of freeze-thaw with the biotin attached.

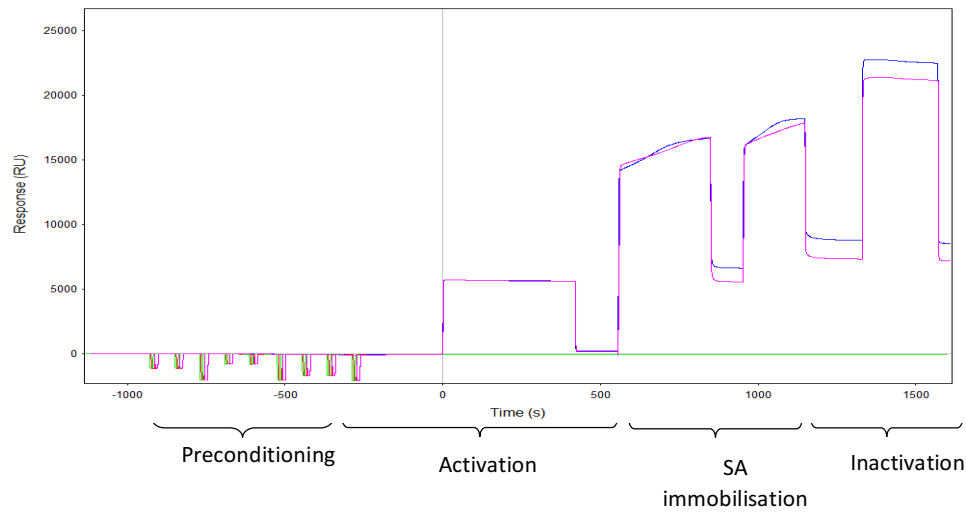


Figure 9: Sensorgram of preconditioning and immobilisation of streptavidin on flow cells 3 and 4 (blue and pink)

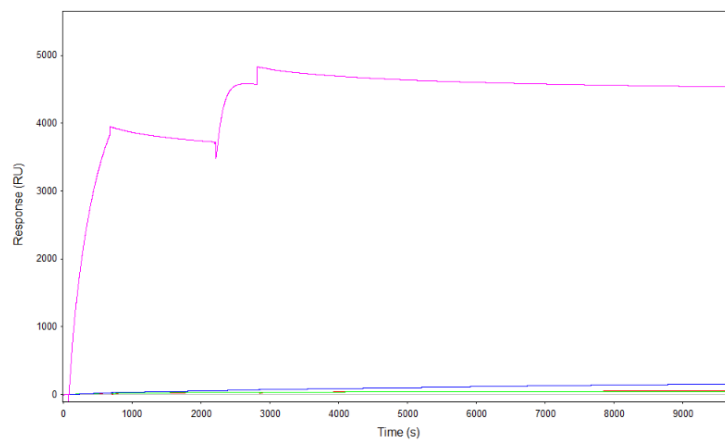


Figure 10: Sensorgram of the capture of biotinylated ITK capture to approximately 5000RU after two injections, first one observed at 0s and second observed at approximately 2500s.

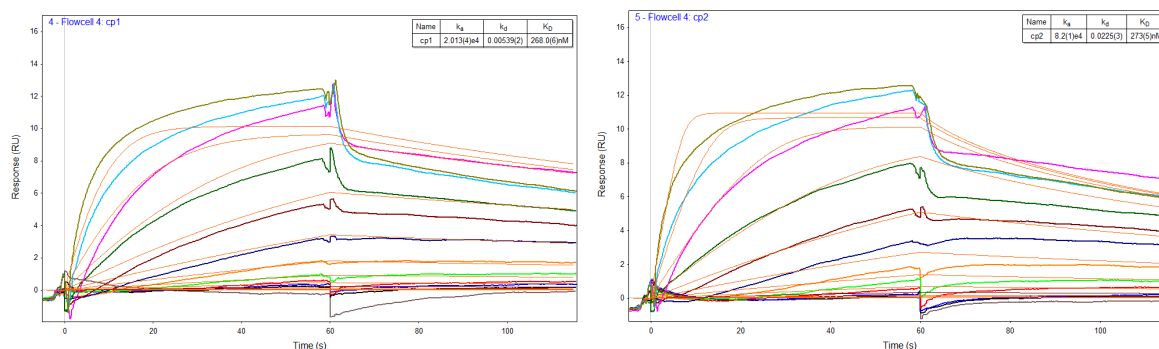


Figure 11: Binding sensorgrams for CP1 injected over ITK surface at concentrations $10 \mu\text{M}$ - 0.0488 nM . The compound shows complex binding profile however is reaching saturation at the highest concentration of $10 \mu\text{M}$.

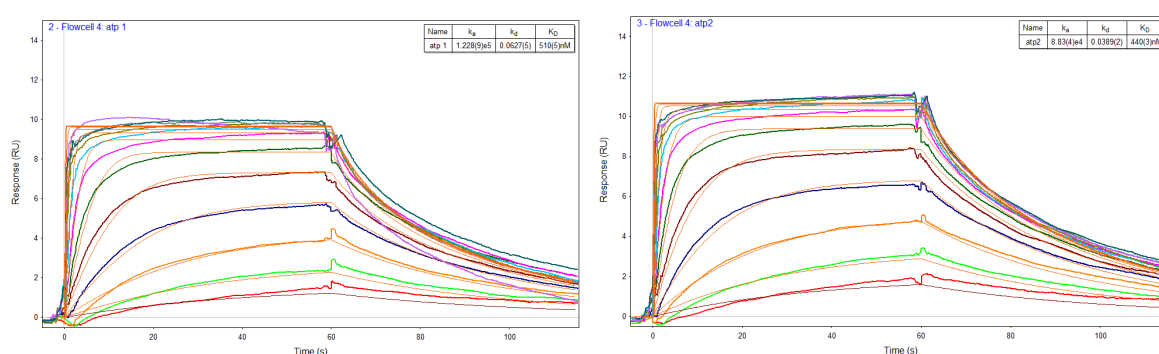


Figure 12: Binding sensorgrams for ATP injected over ITK surface at concentrations $200 \mu\text{M}$ - 0.976 nM .

The allosteric compound by Pfizer effectively bound to ITK, displaying a complex non-1:1 kinetics binding model as seen in Figure 11, which results in a hard to fit model in Scrubber, with possible inaccurate affinity (K_D) as a result. In Figure 12 it can be seen that ATP on the other hand, displayed a typical 1:1 kinetics binding model, hence the data can be easily fitted.

3.2.2 SPR Assay development and optimisation

Manganese influence on protein activity

For this experiment, neutravidin was immobilised instead of streptavidin for capture of biotinylated ITK (Figures 13 and 14), and the running buffer excluded manganese. Neutravidin was proven to be slightly less efficient at capturing biotinylated ITK, although this variability may be due to experimental conditions. As a consequence, SA capture of biotinylated ITK was chosen over NA for the remaining experiments.

The presence of Manganese, which has been shown to improve binding interactions in other studies (*Lovitt et al., 2010*), has been observed to improve slightly the quality of ATP binding (figure 15), although had no effect on Cp1 binding (Figure 16). The improvement can be seen by the increase of the registered Rmax of ATP, in contrast with the similar Rmax of Cp1.

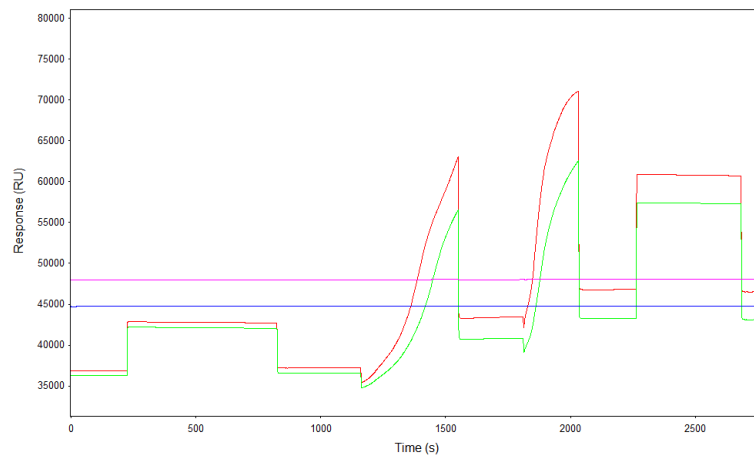


Figure 13 Activation of the surface of flow cells 1 and 2 followed by Neutravidin immobilisation and consequent blocking of the surface with Ethanolamine. NA was injected twice to achieve desired capture level, of a minimum of 5000RU and a maximum of 8000RU. The capture level was close to 7000RU.

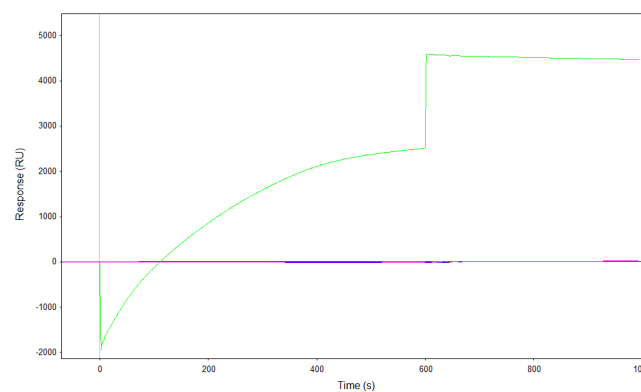


Figure 14: Capture of biotinylated ITK on NA surface on flow cell 2. Capture level reached approximately 5000RU after two injections of ITK.

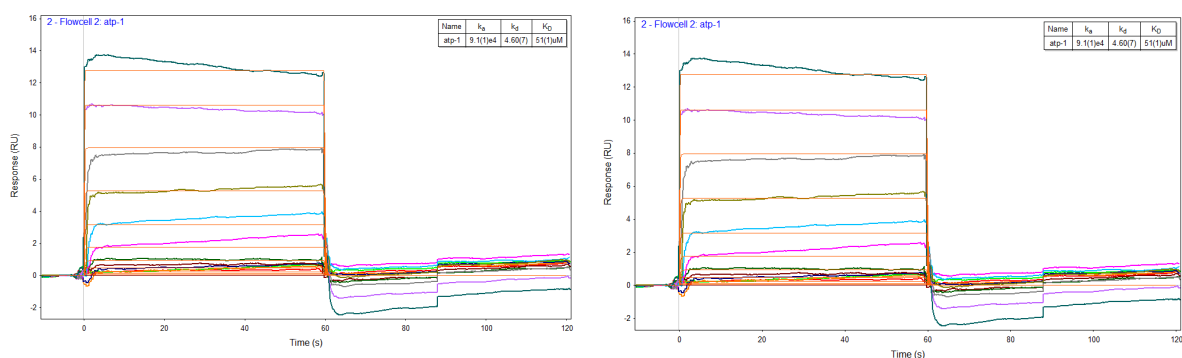


Figure 15: Binding of ATP in the absence of Manganese (right) and the presence of Manganese (left). The R_{max} recorded is higher than the one in the absence of Manganese.

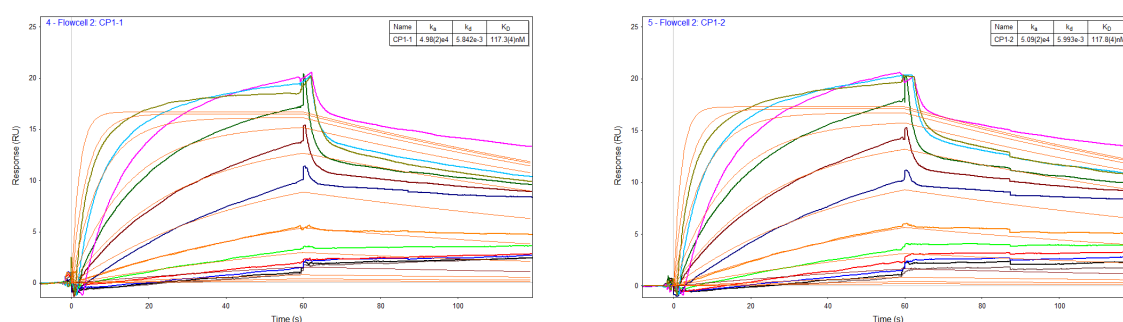


Figure 16: Binding of Cp1 in the absence (right) and presence (left) of Manganese. The manganese presence had negligible effect on the R_{max} achieved with Cp1.

Validation of allosteric activity

An assay to confirm the allosteric activity of the Pfizer allosteric inhibitor was performed in T200. Running buffer was supplemented with $1\mu\text{M}$ BMS compound to block the orthosteric site of ITK. Cp1 showed response to allosteric site of ITK, no binding of ATP was observed (figure 17).

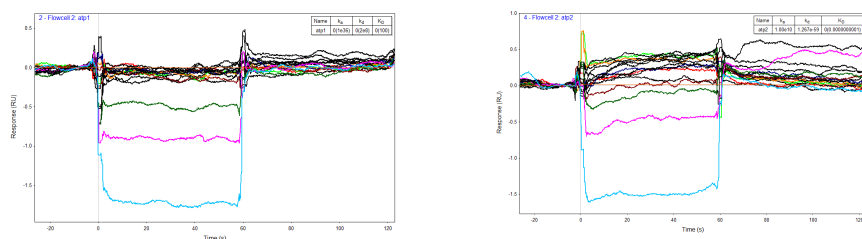


Figure 17: ATP showing negative to no binding on the surface. This is due to the BMS inhibitor blocking the orthosteric site, which displaces ATP from binding, which shows the effectiveness of BMS inhibitor as an orthosteric blocker preferable from ATP in a fragment screen.

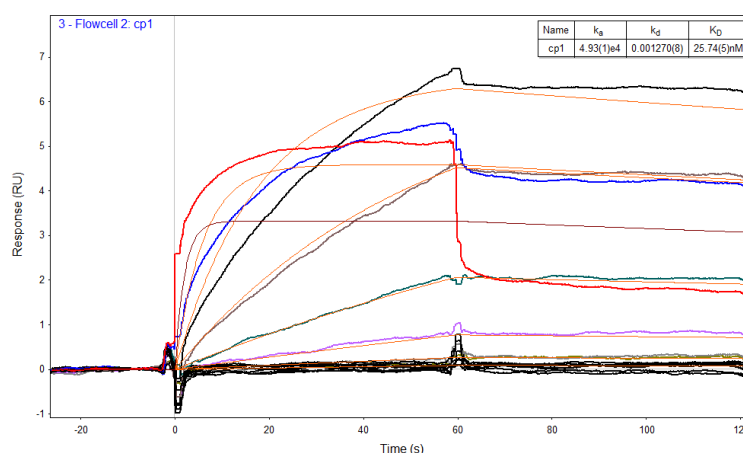


Figure 18: *Binding of the Cp1 to the allosteric site. The binding kinetic profile is complex, with R_{max} being achieved at a lesser concentration than the highest one. Optimisation of the concentrations range is therefore required for a more accurate fit.*

Cp1 exhibits an R_{max} of 8RU in this experiment (Figure 18), nearly half of that seen previously in the absence of a blocking agent in the assay. This is because the mass of the binding agents is directly correlated with the response, and since the orthosteric site is being blocked by the orthosteric inhibitor, it is logical to see half of the response if the Cp1 is only occupying one of the pockets as opposed to two.

Assessment of protein stability: Z prime

The Z prime assay is an assessment of protein stability over time. This assay was performed in T200 and proved ITK to be highly stable over the approximate duration of an average fragment screen, 48h.

Since the experiment was run over two flow cells, one being blocked by the orthosteric inhibitor from Merck, it was possible to once again confirm the mechanism of action of the Merck inhibitor, where it is shown to displace ATP and occupy the binding pocket, and it can be seen contrasting with the ATP injections in the blocked cell, where the binding of ATP to ITK exhibits a very low R_{max} . The following figures (figures 19 and 20) show the controls on the unblocked and blocked flow cells at the beginning (left) and the end (right) of the experiment. The recorded response is lower at the end of

the experiment, which is product of the protein slowly losing activity over time.

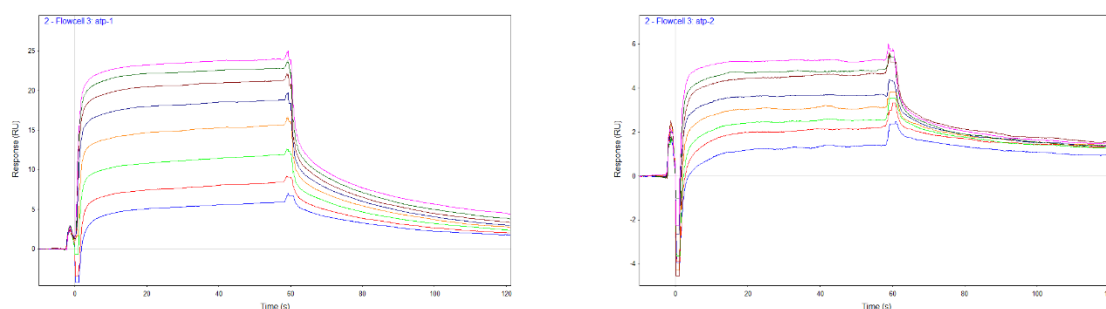


Figure 19: *Flow cell 3 concentration series of ATP, at the beginning and end of the experiment, respectively.*

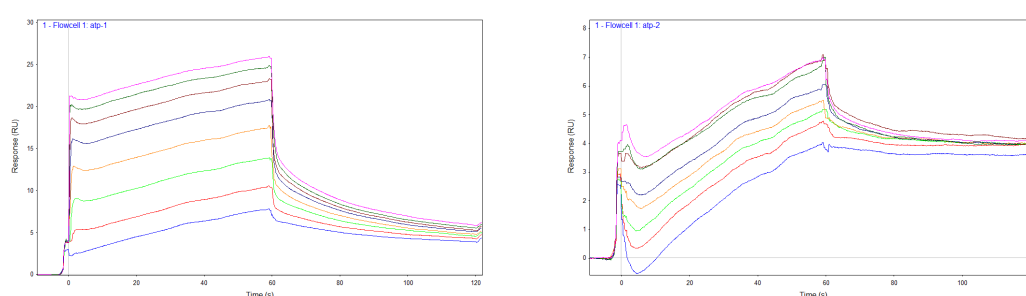


Figure 20: *ATP controls in flow cell 1. The left corresponds to the series at the beginning of the experiment, and the right one to the ones at the end. Decreased response can be appreciated at the end of the experiment as the protein loses activity.*

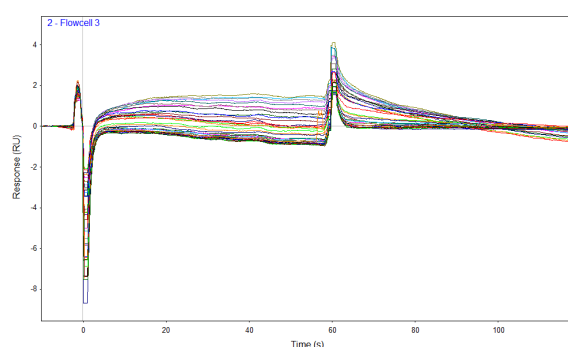


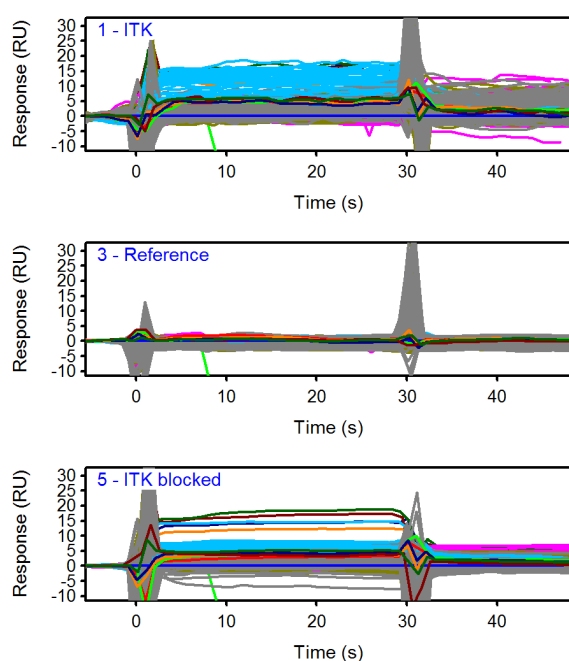
Figure 21: *ATP injections showing little to no response on flow cell 3. This is due to the blocking by the orthosteric compound.*

This assay corroborated the orthosteric binding mechanism of the Merck inhibitor and the displacement of ATP from the active site (Figure 21), and proved that the protein ITK is sufficiently stable for undergoing an average fragment screen.

3.2.3 Fragment screen

A set of 656 fragments obtained from the Drug Discovery Unit at the University of Dundee was successfully screened on ITK using a Biacore 4000. The screen, performed in two days, showed high activity protein levels throughout. The resulting experiment showed several orthosteric binders, although no allosteric binders were found on the blocked flow cell by the Merck orthosteric inhibitor, despite the fact that the library contained fragments with similar structure and overall physicochemical properties to that of parts of the allosteric compound.

Report point



ATP control

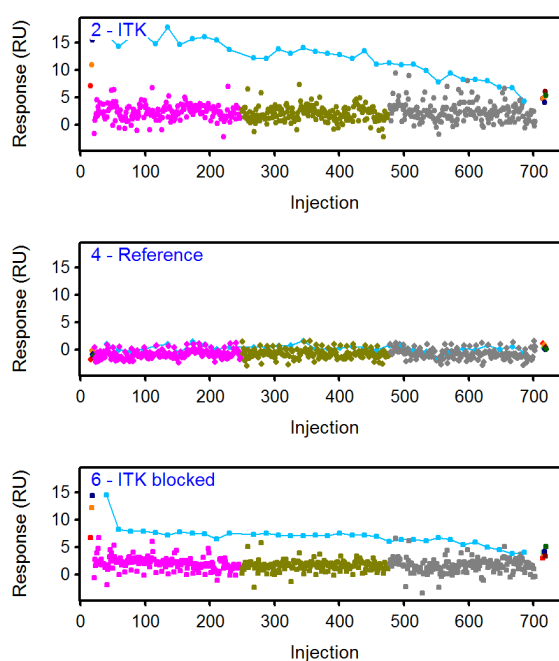


Figure 22: *ITK* fragment screen sensorgrams. The left sensorgrams are a representation of the whole screen, focused on the unblocked *ITK* activity, the reference flow cell, and the blocked *ITK* surface with the orthosteric compound. The blue line represents the binding of ATP, which is seen to be evident on the first sensorgram, nul on the reference, and very low to negligible on the blocked surface. The dots at the bottom of the graph represent each of the fragments, with the colors being: pink at highest concentration, green at middle concentration, and grey at lowest concentration.

The above figure (Figure 22) represents the sensorgram recorded throughout the screen. This fragment screen obtained several successful fragments which were

orthosteric fragments, with some potential hits on the blocked surface that may indicate allosteric activity.

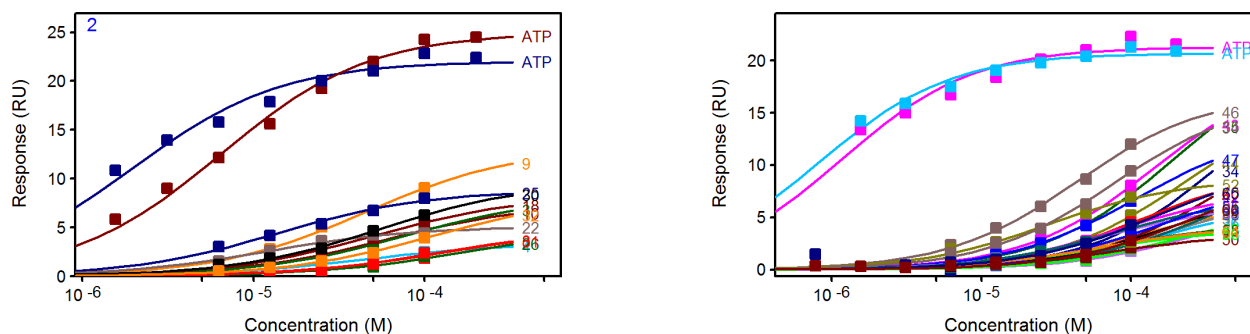


Figure 23: Affinity curves representing the binding of the fragments on the two days of the fragment screen against ITK (left, first day; right, second day). ATP curves correspond to the ATP response at the beginning of the screen (brown on the left and pink on the right) and at the end (dark blue on the left and cyan on the right). The lower curves correspond to the positive binders on each of the screen days, identified by numbers, in a range of concentrations of 13.7-370nM. The second day screen showed an overall slightly higher response than the set analysed on the first day.

The affinity fits of the fragment screen are represented in Figure 23, where the Response units of each fragment, as well as the ATP controls, are plotted against concentration. This is an alternative view of the sensorgram, where the concentration is represented as the defining factor for the response obtained. The affinity fit is an effective way of determining an estimate of the K_D of compounds that exhibit complex binding.

3.2. Src kinase SPR assays and fragment screen

3.2.1. Testing of Src kinase activity

The Src kinase construct was tested against ChemBL orthosteric inhibitor in MASS-1 and its activity was confirmed. The K_D of the construct was of approximately 50nM (Figure 24), which was expected of this compound.

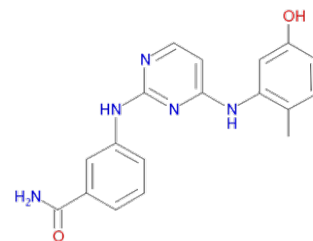
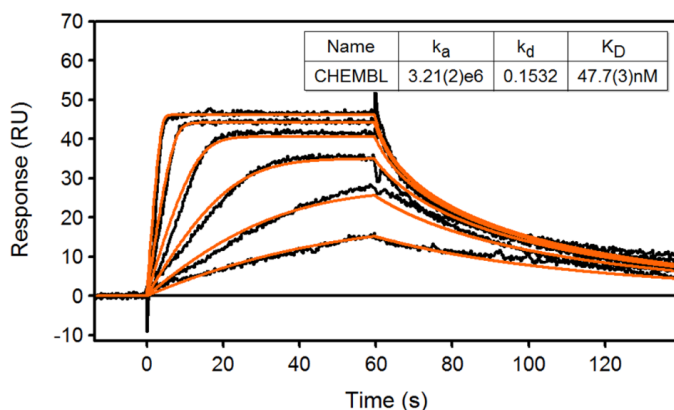


Figure 24: *Src kinase control kinetics*

Injections of concentration series were performed over immobilised target at 25C. The orthosteric inhibitor ChemBL was shown to have high affinity for ITK at ~50nM K_D . Kinetic model was shown to be 1:1, with fast association and moderate dissociation time. Chemical structure of ChemBL control compound is seen at the right of the sensorgram.

The compound employed on this control has inhibitory properties in proteins Yes, Csk and Bmx (*Bamborough et al., 2008*). Yes is part of the Src family kinases, hence this inhibitor was used to test the ability to bind to Src kinase. The activity test resulted positive, with expected binding to the protein. It was used as control thereafter in the fragment screen and the confirmation studies.

3.2.2. Fragment screen

From the fragment screen, several possible binders were obtained. A total of 32 hits were confirmed, and they were selected for confirmation experiments in T200. The ChemBL compound was regularly injected both in fixed concentration (Figure 25) and concentration series at the beginning and end of the assay (Figure 26) to confirm preservation of protein activity throughout the fragment screen.

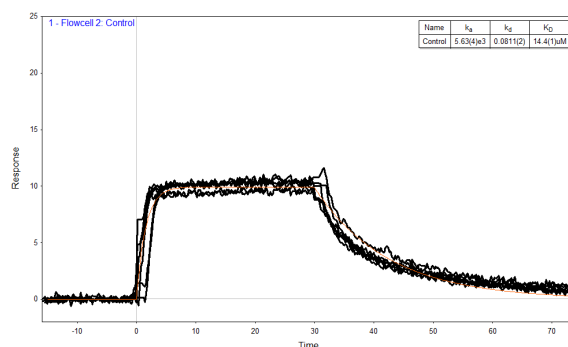


Figure 25 *Sensorgram depicting 1 μ M control injections of ChemBL compound control at the beginning of the assay. This was performed to ensure protein activity and consistency of binding response to the surface.*

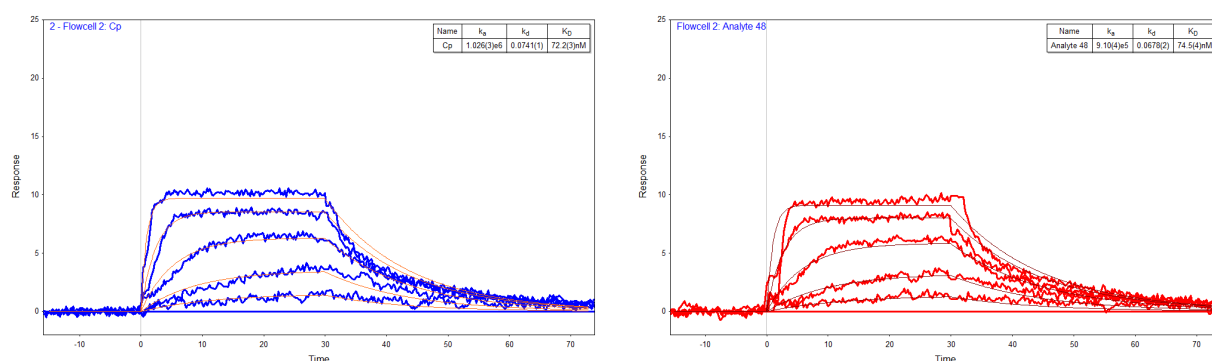


Figure 26: *Injections of ChemBL compound at the beginning (left, blue) and at the end of the assay (right, red). This was performed to ensure protein retains activity at the end of the experiment, and that the response is consistent between the two time points.*

This screen gave as a result several fragments with apparent strong binding to Src kinase, some exhibiting a potentially allosteric activity. A few examples of the fragments obtained from this screen that were further selected for confirmation are shown in Figure 27.

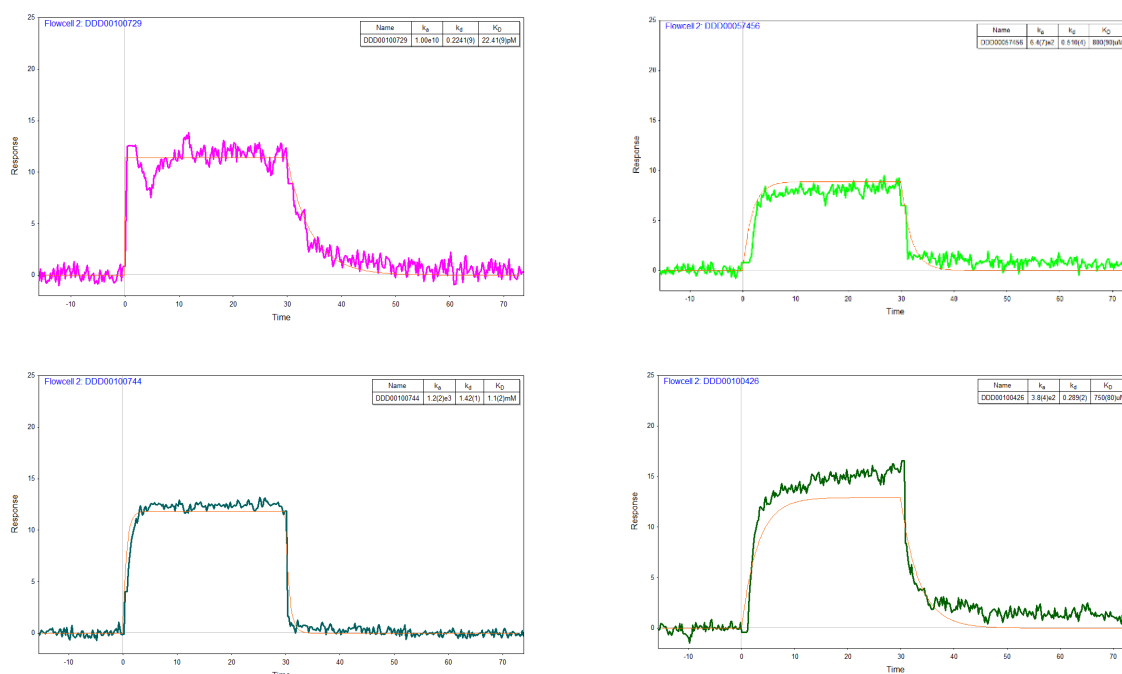


Figure 27: *Examples of binders found at the fragment screen. The fragments that showed this kind of potency were selected for the confirmation studies.*

3.2.3. Confirmation studies

The selected fragments were subsequently screened in T200 against Src kinase kinase. The resulting assay revealed several fragments with potential allosteric activity.

For control, Dasatinib was tested in Src kinase as orthosteric inhibitor. It is a potent kinase inhibitor with a slow off-rate, which is a desirable characteristic for a control in this experiment, since it provides effective blocking of the orthosteric site in the kinase (*Li et al., 2010*). Dasatinib, used as control, provided effective blockage of the surface at 1 μ M injections. Control series were also performed to evidence the kinetics and the R_{\max} of the compound. One flow cell was blocked with Dasatinib and another was not, to contrast allosteric binders to general binders. The concentrations series of Dasatinib can be seen on Figure 28 and the 1 μ M concentration control injections of ChemBL are illustrated in Figure 29.

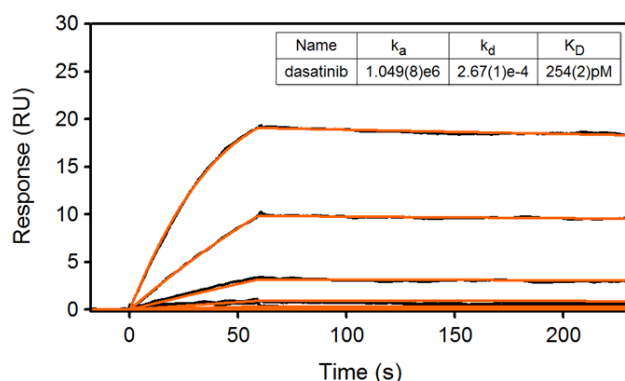


Figure 28: *Dasatinib control series. 3 fold concentration series from 56pM to 41nM, included to ensure kinetic profile of Dasatinib is constant, and the experiment reproducible.*

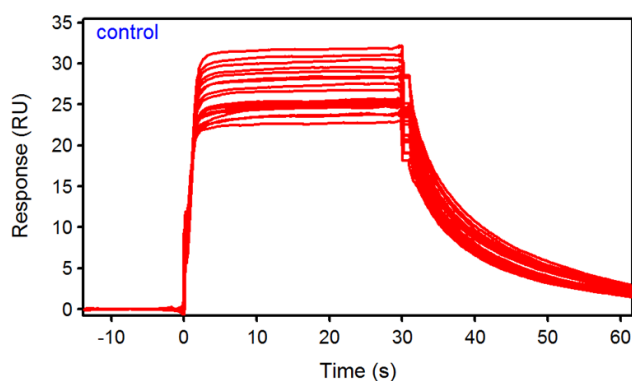


Figure 29: *1 μ M control injections of ChemBL at the start of the assay.*

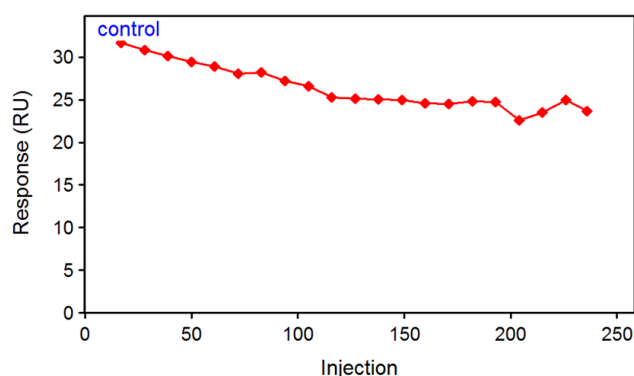


Figure 30: *Representation of the Response units at designated time points of the ChemBL control in SPR experiment of confirmation, representing activity (RU) vs time (injection time point at seconds).*

Compound hits screened at 1.85-150 μ M concentration series, at three concentrations, were screened in two flow cells in a competition study, consisting of the surface possessing captured native Src kinase over two flow cells, with one flow cell being blocked by Dasatinib constant injections at 1 μ M. Multiple binders were obtained, many in the absence of Dasatinib blocking (figure 30). Fragments that would show a binding profile on the presence of Dasatinib (figure 31) would be likely to have an allosteric mode of binding. This is an effective preliminary way of determining which fragments are allosteric.

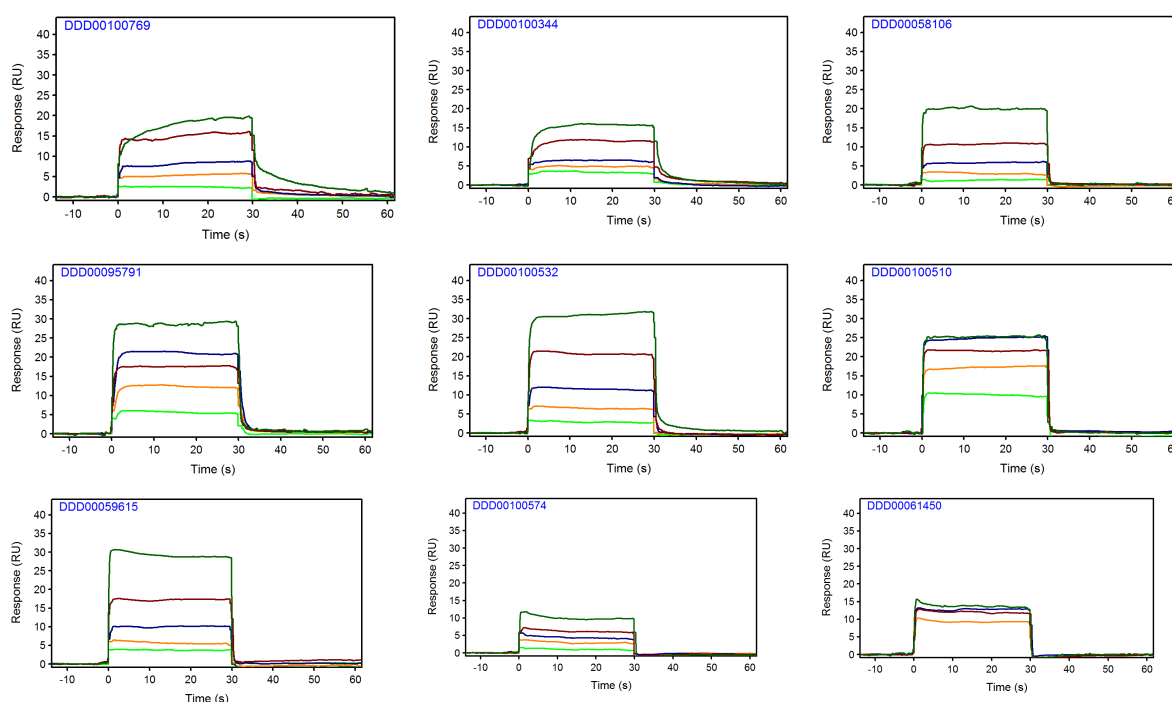


Figure 31a: *Example of several compound hits, binding in the absence of Dasatinib.*

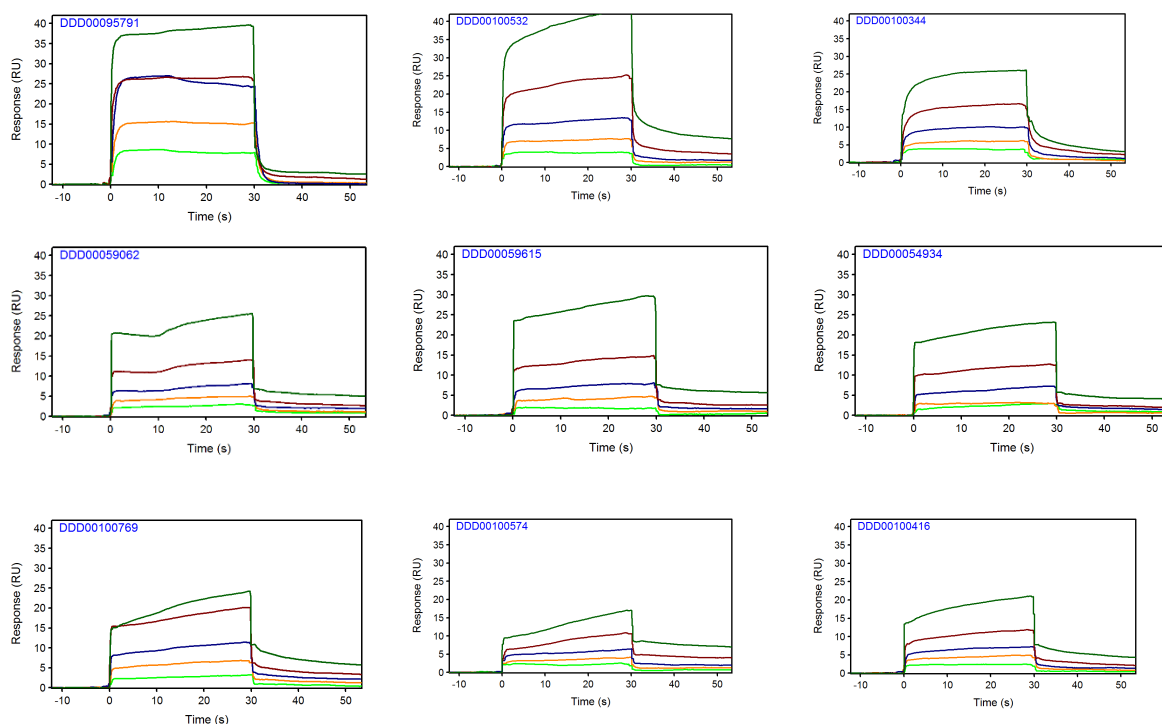
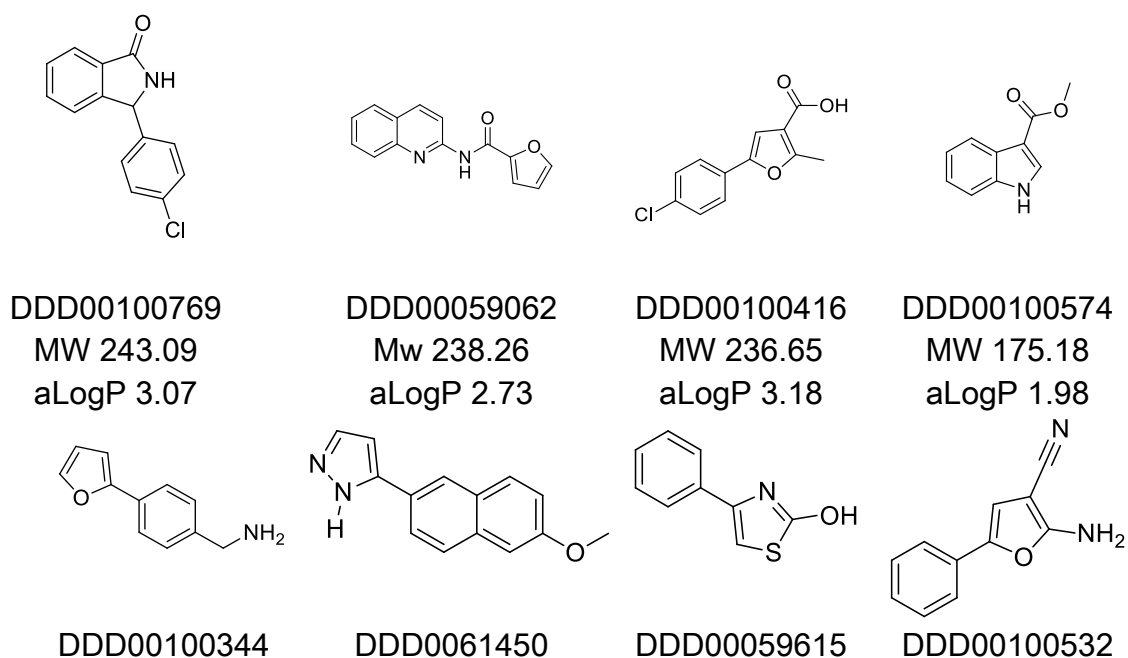


Figure 31b: *Compound hits in the presence of 1 μ M Dasatinib. These fragments are likely to have allosteric activity since they show binding despite the blocking of the orthosteric site by the control.*

The chemical structures of the fragments that bound to the Dasatinib-blocked Src kinase are represented below in figure 32. These hits are highly likely to be allosteric binders.



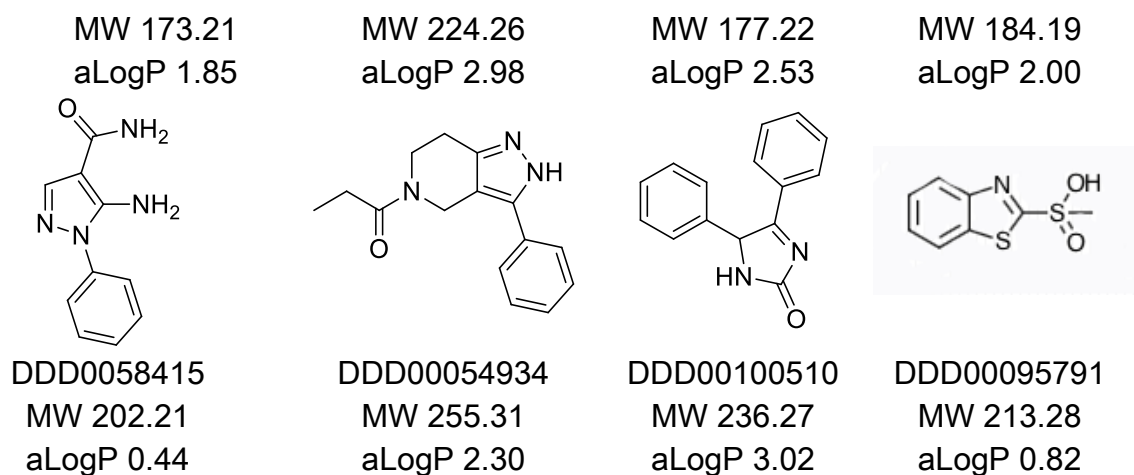


Figure 32: *Chemical structures of binders to Src kinase in the presence of Dasatinib. These compounds have a high potential of being allosteric binders, although further kinetic studies are needed to confirm this.*

3.3. Computational studies

The following figures (33 and 34) are the alignments performed with Jalview. The proteins analysed were protein tyrosine kinases, phylogenetically related to ITK and the TEC kinase family. These were selected as a representative of each family, in a random fashion. The first alignment performed included only the TEC family of kinases, and the allosteric pocket was identified by means of identifying the surrounding residues of the allosteric binding site, and mapping the interactions of the residues with the inhibitor. These interactions were then reduced to a range of 4 Angstroms, and the individual amino acids were consequently identified. The type of interaction was also mapped, and the ones that had direct contact with the inhibitor were isolated. These residues were the basis of the allosteric pocket reference from ITK and were used as a template and guide in the rest of the alignment.

The alignment ran with a ClustalW algorithm. It shows a considerable percentage of conservation for the TEC family of kinases, fact that is expected due to their phylogenetic resemblance. Figure 40 corresponds to the first half of the pocket, where the domain was coloured by using a BLOSUM62 matrix, by percentage of conservation, where the darker shades corresponds to a higher conservation match. Figure 41 shows the second half of the alignment, on the region that contains the DFG motif, common to all protein kinases. The Asparagine and Phenylalanine of this motif is shown to have a direct interaction with the Pfizer inhibitor in ITK, hence it has been highlighted as part of the pocket. This motif is ubiquitous throughout the kinases studied, possessing an essential role in regulating the conformation of the kinase.

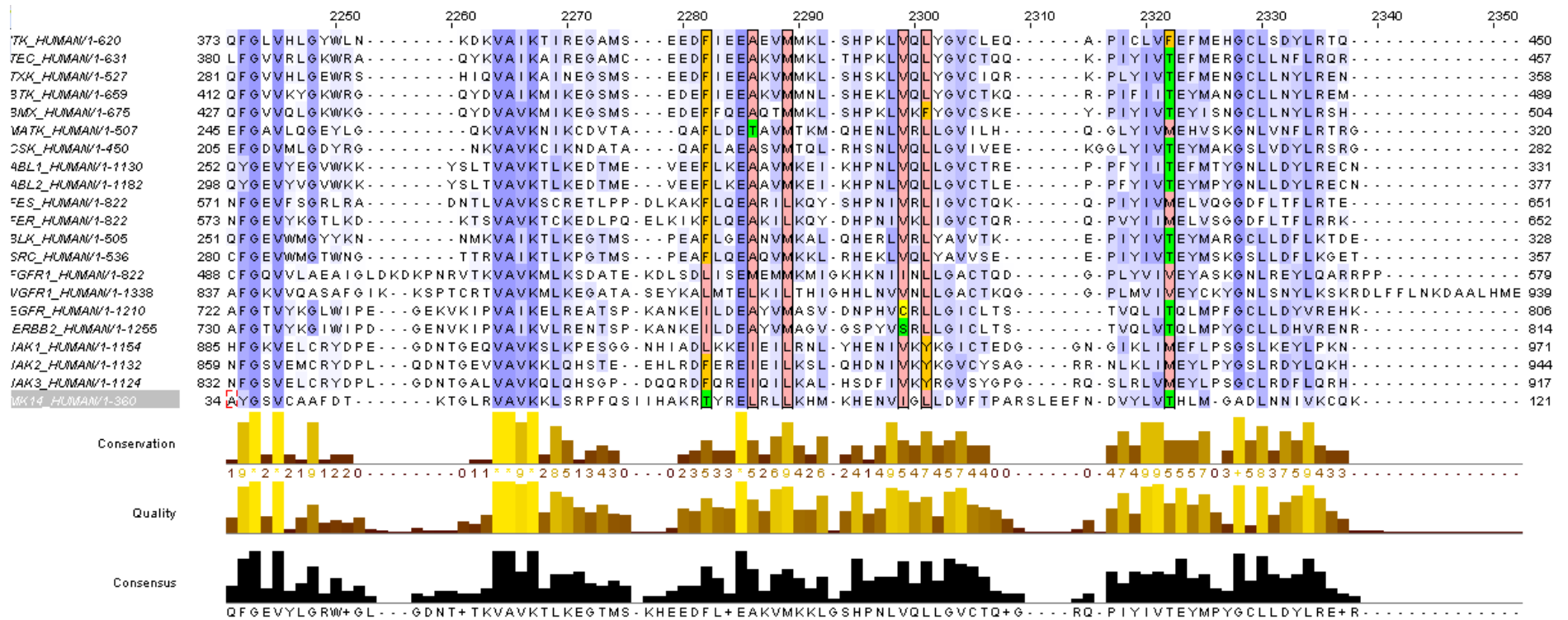


Figure 33: Alignment of the selected Tyrosine Kinases using JalView. The algorithm selected was T-Coffee. The highlighted residues were those found to bind to the allosteric site in ITK, and they were colour-coded using Zappo colour scheme for identification of their physic-chemical properties.

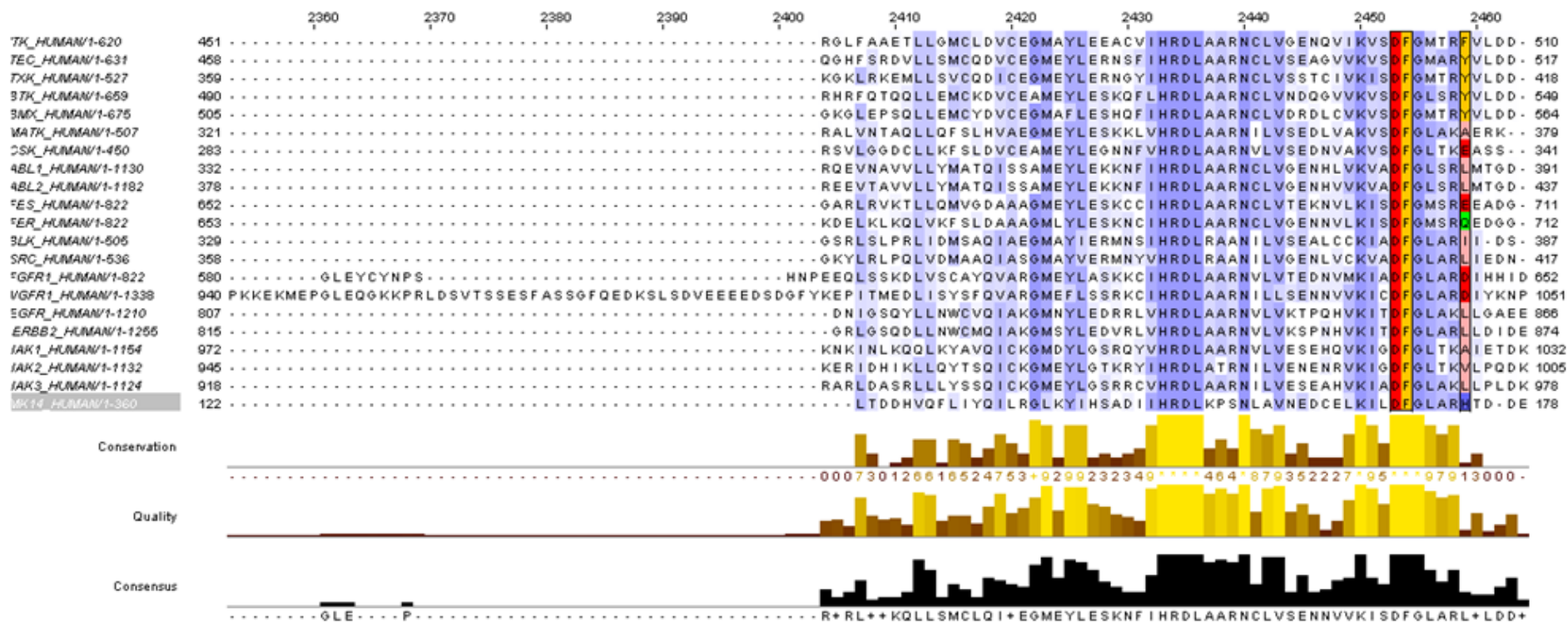


Figure 34: Continuation of the alignment of allosteric pocket taking ITK as a reference. In this figure, the DFG motif is seen, with the D and F residues being 100% conserved throughout. These are known to interact with the ITK allosteric compound, although not the Glycine of the same motif. The DFG motif acts as a regulator of kinase activity, being ‘flipped’ outwards when an allosteric ligand interacts with the kinase in the pocket. This factor can be determinant in the design of novel allosterics.

4. Discussion

4.1. Computational studies

4.1.1. Analysis of the alignment & Discussion

The first residue from the ITK allosteric pocket, Phe 403, was identical not only throughout the TEC family members but also in MATK/CTK, CSK, ABL1, ABL2, FES, FER, FRK, BLK, SRC, JAK 2 and JAK 3. JAK1 had in that position a Leucine instead of a Phenylalanine. The remaining kinases had also an amino acid with hydrophobic properties, be it a Leucine or an Isoleucine.

The second residue, Ala 407, was also predominant in the TEC family of kinases, and at that site it appears that hydrophobic residues are the rule, with the rest of the kinases exhibiting either methionine, leucine or isoleucine. The exception to this is CTK/MATK, which has a Threonine at that site. This was unforeseen since Threonine is a strongly nucleophilic/hydrophilic amino acid.

Met 410 is highly conserved, even in the distinct EGFR1. Where a methionine is not present, a leucine is present, an amino acid with similar properties to methionine. This suggests the presence of a strongly hydrophobic pocket as in ITK.

The next three residues, Leu 413, Val 419 and Leu 421 were also highly conserved throughout the alignment with the strongest exception of serine and cysteine in the Val 419 in EGFR1 and ERBB2. For the site corresponding to Leu 413, tyrosine residues were present for FES and FER. The less hydrophobic nature of the Tyrosine gives rise to the doubt of the overall hydrophobicity of the pocket and the feasibility of an allosteric site for these enzymes, with a similar problem for Leu 421 in the JAK kinases.

For Phe 435, the results showed there is more similarity among the rest of the kinases than with ITK in the sense that the only kinase with a phenylalanine at that position is ITK, the rest are either Threonine or Methionine. Given that this Phe 435 is key in the interaction with the compound discovered by Han et al., it rises questions as to what the shape of this pocket would be in the rest of the kinases.

The most unpredicted finding from this alignment was that the residues Aspartic acid 500 and phenylalanine 501 are conserved 100% throughout the kinases analysed. This is something worth noting since a compound with interactions that can function as an electrophile and can induce pi-stacking with the phenylalanine, such as the one reported by Han et al., may possess potential as inhibitors. This, coupled with an analysis of the potential pharmacophore backbone for each of the kinases, may lead to powerful probes to assess the presence of allosteric pockets in these kinases. In

the case of Abl1, it was particularly interesting to find such a strong similarity in the ITK allosteric site. Abl1, which has been shown to have a regulatory allosteric binding pocket known as the Myristic binding pocket, could possibly have this second pocket as an allosteric site.

4.1.2. Exploration of the ITK binding site in PyMol

In order to ascertain the pharmacophore and the essentiality of the residues in the ITK allosteric site towards the binding of the inhibitor, an in-depth analysis of the crystal structure of ITK reported by Han et al. (PDB code 4M0Y) was studied in PyMol. The residues participating in the binding were isolated, and with the help of web-based java application Ligand Explorer, the polar and hydrophobic interactions were drawn. The gatekeeper residue Phe 435 proved to only have one distant hydrophobic interaction with the inhibitor. The presence of a threonine in place of this phenylalanine on some of the kinases gives hopes of polar interactions for the designed probes using an analogous scaffold as the ITK allosteric inhibitor.

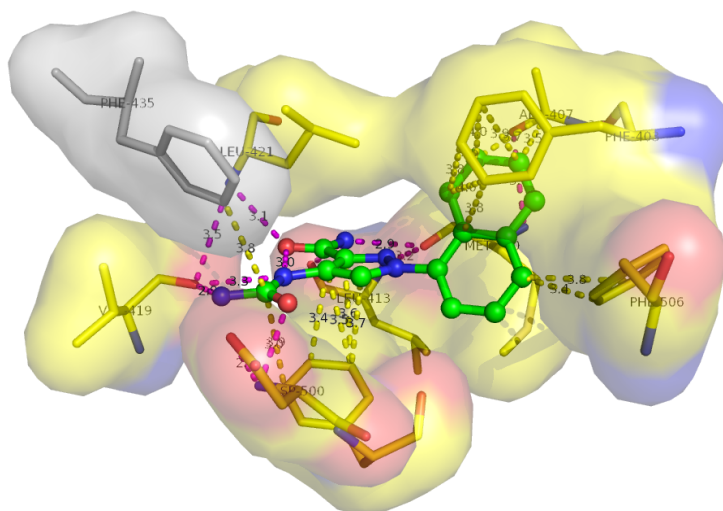


Figure 35: overview of allosteric site in ITK. The magenta dash lines represent the polar contacts and the yellow dash lines represent the hydrophobic interactions.

The allosteric pocket and all the interactions are shown in Figure 35. As it can be seen in Figure 37, the majority of the interactions are hydrophobic. The double ring system in the ITK inhibitor results in a buried pi-stacking interaction with the residue 403, a phenylalanine, and this is highly conserved in the TEC family of kinases (ITK, TECK, TXK, BTK and BMX), as well as MATK (CTK), ABL1 and 2, FES, FER, BTK and SRC. On the rest of kinases, hydrophobic residues such as leucine and isoleucine, except on the JAK2 and JAK3 kinases, where a phenylalanine is found once again. p38 differs

from this in that it has a threonine, a polar residue, at this position. The main concern in this alignment resulted from the fact that Phe435, a key amino acid in ITK, is not conserved throughout the alignment. At this position, most of the amino acids are threonine, valine or methionine.

The analysis of the crystal structure using both Ligand Explorer and PyMol has shown that only one loose hydrophobic interaction takes place between Phe 435 and the inhibitor (Figure 36), therefore it is likely to be not essential for the binding of the compound. This phenylalanine is also quite removed from the pocket itself, therefore other amino acids would be unlikely to be affecting the folding of this region into a pocket.

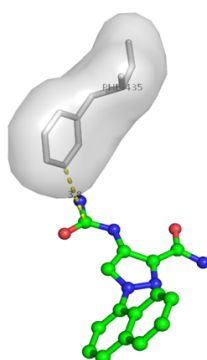


Figure 36: Interaction between the gatekeeper residue Phe435 and ITK allosteric inhibitor

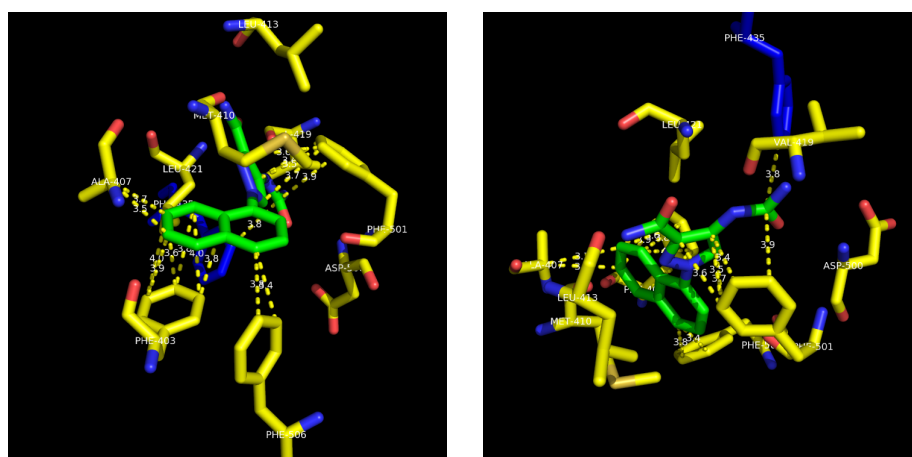


Figure 37: View of the hydrophobic interactions of the binding site with the inhibitor in ITK allosteric site

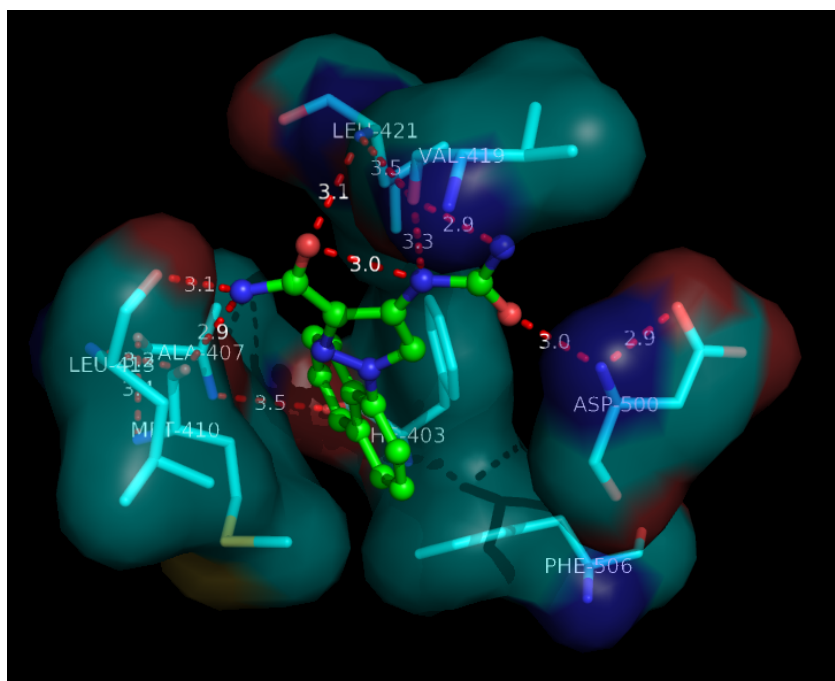


Figure 38: View of the ITK allosteric binding pocket, with corresponding electron density maps, and the polar interactions (red dashed lines) with the allosteric inhibitor. The distance of these bonds is represented in Angstroms, with the average distance being of 3.1Å, which typically corresponds to Van-der-Waals interactions.

The asparagine from the DFG motif has a single polar interaction type of bond that is in the range of a hydrogen bond (Figure 38), and the overall polar interaction map is displayed in Figure 39. Since the polar interactions calculated by PyMol take into account intramolecular interactions, these are also shown.

The existence of two H-bond donors and two acceptors proves very efficient for the binding to ITK. Leucine, valine, methionine and asparagine residues are the ones that form the polar anchoring of the molecule to the target. In order to further develop a ligand for this pocket, the strong polar interactions of these functional groups with the target are worth considering. Perhaps lengthening one of the chains with an extra carbon may shorten the distance between the polar residues and the ligand, therefore giving rise to a stronger bond in the 2 Angstrom range. The middle ring does have some interaction with the hydrophobic residues, but the most significant hydrophobic interaction is the double ring system that is used as an anchor and pi-stacks with phenylalanine. This one perhaps should be conserved or one ring could be removed at most if faced with solubility issues.

4.1.3. Chemical breakdown of the allosteric inhibitor of ITK: essential structures and proposed modifications

After what was learned from the modelling in PyMol, questions as to what the pharmacophore would be arose. The most probable option is that it lies primarily between the double ring system functioning as a hydrophobic anchor and the polar sites at the opposite side of the molecule, as shown in Figure 39. Two “tails” with polar heads, as this molecule has, is highly effective in binding the residues in the allosteric site. Hence, the following schematic was generated using ChemDraw Ultra (Figure 39). Several suggestions for fragments with allosteric activity were designed parting from the knowledge gathered from the structural studies conducted.

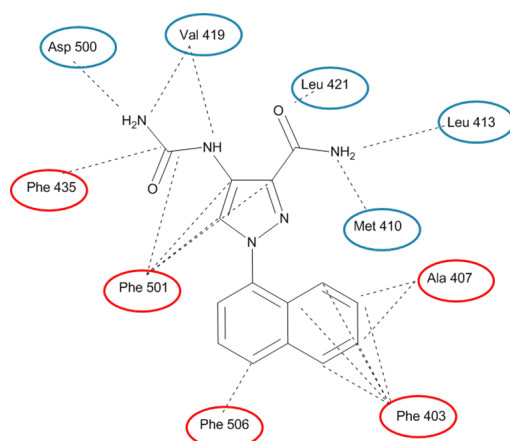


Figure 39: 2D map of the interactions of the Pfizer allosteric inhibitor within the allosteric pocket of ITK. The hydrophobic interactions are highlighted in brown, and the polar bonds in blue.

Within the TEC family of protein kinases, there is one that has major implications for current therapies: Bruton's Tyrosine Kinase (BTK). BTK is the only TEC kinase whose deficit directly implicates a disease. This one is the rare X-linked agammaglobulinemia. This enzyme might also be implicated in Rheumatoid arthritis, a serious condition affecting the joints of autoimmune origin and unknown aetiology. Since there is a crystal structure of BTK's kinase domain, this was aligned with ITK in PyMol and the potential allosteric site was compared, as seen in figure 40 where the two proteins were aligned and overlapped on their kinase domain, and the allosteric site was selected. It can be observed that the Pfizer inhibitor does not clash with BTK, and in fact exhibits a similar fit than in ITK.

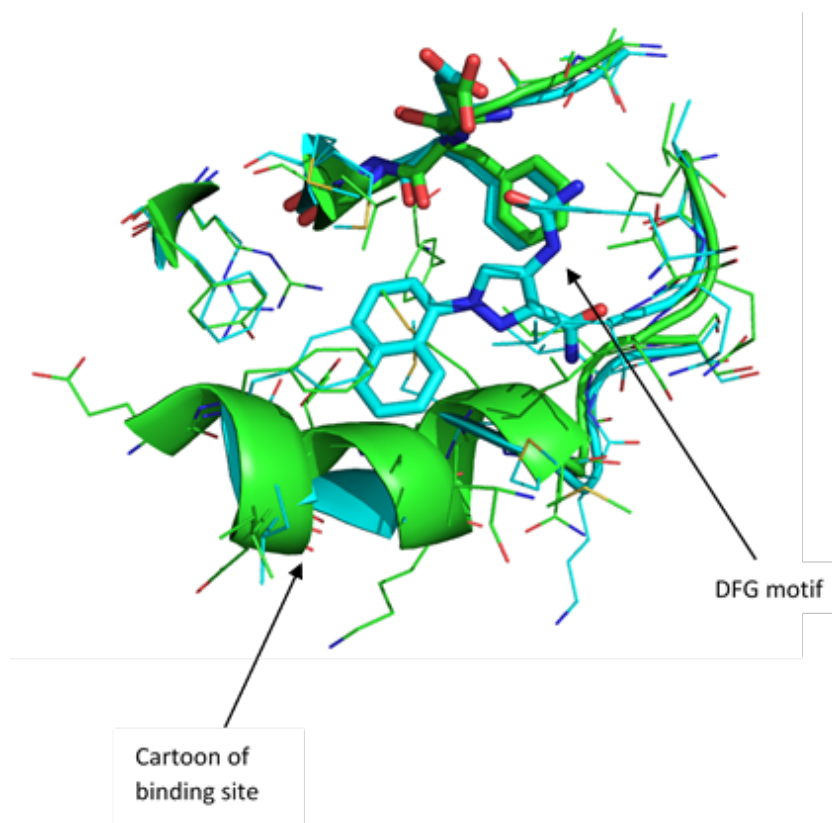


Figure 40: Aligned and superimposed structures of ITK and BTK kinase domains. ITK is in blue and BTK in green. The ITK allosteric inhibitor is shown to fit similarly in both pockets.

This analysis was performed to see if theoretically probing BTK with the allosteric inhibitor would be effective. This is exciting since there is a probability that this method can be effective.

From this study it can be concluded that there is a significant possibility that an allosteric pocket can be found in protein kinases. It has been seen that throughout the tyrosine kinases analysed the possibility of having similar residues participating in the binding of the potential inhibitors may be similar than that to ITK's, although different enough as to provide selectivity, contrasting with the ATP site.

4.2. ITK Assays

ITK was successfully cloned, expressed and purified at first try, without need to further optimise the protocol. The expressed construct was stable and durable, retaining activity despite the freeze-thawing, and the chemical biotinylation procedure, which involves a 2h ice incubation, and would not be viable with a less stable protein. The immobilisation via Streptavidin on a CM5 chip proved reproducible and reliable, and

the capture levels averaging 5000 RU were sufficient for the correct performance of the assay.

The allosteric binding of the Pfizer inhibitor Cp1 was confirmed, and the nature of the binding can be seen as bitopic, since when the orthosteric site was blocked by the Merck orthosteric inhibitor, the R_{\max} was half of the reported one in its absence. This is due to the fact that RU are directly related to the mass on the surface, hence the molecules of Cp1 binding to each immobilised ITK would be half due to their displacement from the orthosteric site by the Merck inhibitor, here called BMS. It has nanomolar affinities for ITK, and exhibits a non-1:1 kinetic binding model. This made it difficult for the data fitting, since we had to take into consideration the presence of Mass transport and its constant, and it challenged the extraction of accurate kinetic data from this experiment. To optimise this and eliminate the mass transport, the concentration of ligand should be increased to a maximum of 10 times its observed K_d , so that the ligand is saturated on the surface and ensures maximum binding.

The SPR assay was developed and optimised for ITK in order to prepare for fragment screening. The ideal conditions for this were: the presence of Manganese in the buffer, the pH was kept at 8 and was suitable for the stability of the protein, since there was no evidence of drift or denaturation on the surface.

The results from the fragment screen yielded many strongly binding fragments, and in the confirmation studies, there was a presence of orthosteric binders. However, there were no reliable hits for allosteric binders. The hits that were found to be responsive in the presence of the blocking of the orthosteric inhibitor from Merck were not reliable. Possibly the chemical space represented by the relatively small fragment library is insufficient to probe the specific interactions needed to perturb the allosteric binding site.

A further study to confirm these fragments do bind to the protein in the presence of an orthosteric inhibitor would be appropriate, to extend the range of concentrations of these fragments. Enzymatic studies and ITC would be other alternatives to ascertain the allosteric activity of these fragments.

An alternative would be to change the fragment library and perform these experiments again with a different set of fragments. A proposition to ensure allosterically active fragments would be to synthesise a set of compounds based on the allosteric compound from Pfizer, obtaining the crucial structures within the inhibitor, fragmenting it, and performing variations to create a larger diversity of chemicals.

The fragmentation of the compound would be based on the three chemically distinct structures within the inhibitor: the lower tail end with the double-ring system that likely acts as a hydrophobic anchor within the pocket, the middle part with the amphiphilic properties of the ring system and the nitro-ring, and the urea-derived part of the top, which confers the polar side of the inhibitor.

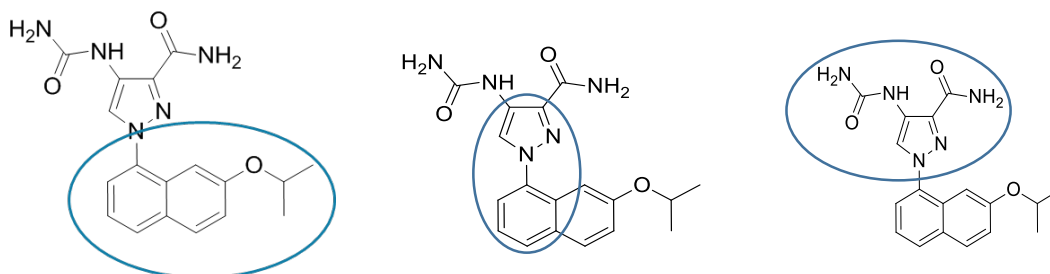


Figure 41: *Fragmentation areas of the Pfizer inhibitor. Each of the structures are circled in blue and would be the templates for the fragment design.*

Commercially available fragments that are shown with structural similarity to the designated fragments have been found, and classified into two groups: hydrophobic fragments (Figure 41) and urea-derived ones (Figure 42).

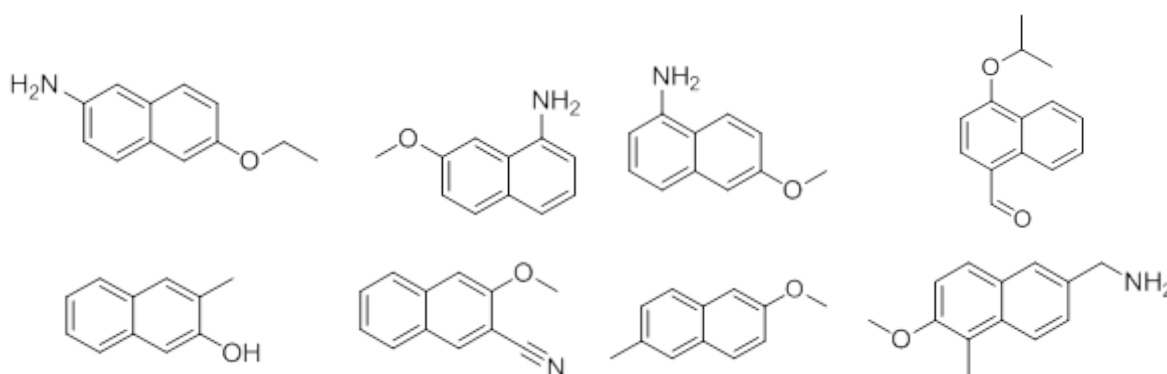


Figure 41: *Commercially available hydrophobic fragments*

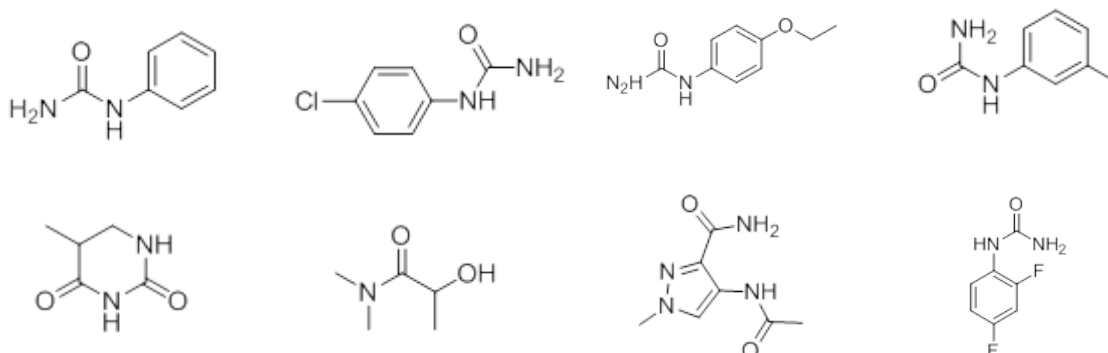


Figure 42: *Commercially available urea-derived fragments*

This new library would have high chances of being allosterically active in ITK and would have the potential to improve the current Pfizer inhibitor. With the new functional groups added, the conformational changes within the allosteric pocket and the interaction with the new fragments, new conclusions could be drawn as to the allosteric pocket affinity and characterisation.

4.3. Src kinase SPR experiments

Src kinase was a stable kinase which was conveniently directly immobilised via amine coupling. The protein was active, as evidenced by the binding of the ChemBL orthosteric compound and the kinetic studies on it. The fragment screen was successful, with the whole assay performed on MASS-1, and the confirmation assay performed on T200. The fragment screen yielded several fragments that showed binding in the blocked surface, hence they were taken forward for confirmation. The assay further ensured the possible allosteric activity of the fragments, with high recorded R_{\max} . These fragments are most likely binding to the allosteric pocket of Src kinase, although where that pocket is located within the protein is unknown, with the exception that it is within the kinase domain. Possible binding sites which would be non-specific would be to the surface or to a partially unblocked orthosteric site, although given that the ChemBL compound was verified to bind to the active site in the kinetic assay, this is improbable.

To ensure these results correspond to the fragments having allosteric activity, further kinetic studies should be performed. To discard the possibility of non-specific binding to the kinase, the kinase could be captured via His tag or biotin tag onto the surface to ensure the directionality of the protein has the catalytic domain exposed, hence the binding is truthfully in the kinase domain and the protein is being properly blocked. Once the allosteric binders had been confirmed as having that mode of action, the ideal step would be to crystallise the protein with the fragments and ATP or ChemBL compound as part of the incubation solution, to observe the location of the allosteric pocket and the conformation that Src kinase adopts.

5. Conclusions

From this study it can be concluded that a detailed analysis of the binding site of protein kinases can enlighten the search for allosteric sites. The fact that the kinase domain is highly conserved throughout the kinome, and that within a kinase family there is a high conservation, is both challenging and useful: it has been found that although the residues that were taken from ITK as a reference for this analysis were not highly conserved throughout the alignment, the physico-chemical properties of these were very similar in the immediately related kinases, and had strong similarities in other families. This is a useful fact in the search of allosterics: the pockets do not possess the same sequence of amino acids as the orthosteric site does, nevertheless the properties of these residues are similar, hence highly likely to form into pockets like that of ITK. The issue of selectivity would consequently be solved with the different residues: allosterics can be tailored for each enzyme, and be less likely to affect others. Further work on this would include the actual testing of these findings experimentally, and observing if the possible pockets are indeed active and druggable.

The results on the Src kinase are encouraging for further study, and give a glimpse of the possibility of identifying and targeting the allosteric site for therapeutic and functional research.

The question that project asked, can we rationally identify allosteric pockets in protein kinases, and effectively target them by the use of fragment screening using a multidisciplinary approach, has a mixed answer: it is clear that the use of computational methods to study the structure and sequence of a protein can offer significant insight of the possible new therapeutics and where they might be targeted to, and it is especially relevant for the study of allosteric pockets, since they are often masked and not so obvious to the eye. If refined enough, this can potentially be used to effectively identify tell-tale signs of a pocket, and the possible kind of chemical that could be used to occupy it. The use of SPR as a biophysical technique to preliminarily assess the suitability of a compound and its mode of action is clearly effective. The modern technologies applied to this technique can provide numerous amount of data in a semi-high throughput scale, with machinery such as Biacore 4000 and Sierra MASS-1 to effectively deliver over 700 fragments screened in less than 50 hours.

This is highly convenient to obtain valuable information on the kinetics of a protein, and to optimise and assess the most favourable conditions for further experiments, including X-ray crystallography and NMR.

6. References

Figure 1 was self-generated, inspired by the diagram found at Cooper MA, Nature Reviews Drug Discovery 2002, 1:515-528.

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